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PRINCIPAL INVESTIGATOR: **Maryam Foroozesh, Ph.D.**

CONTRACTING ORGANIZATION: **Xavier University of Louisiana, New Orleans**
 New Orleans, LA 70125

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14. ABSTRACT Xavier University of Louisiana is in the unique position of developing capability in drug discovery especially in the areas of cancer and health disparities. A significant proportion of the funded research on Xavier's campus including collaborative projects involving Tulane University are related to cancer, drug design, synthesis, and drug delivery. This project **expands the partnership between Xavier University and Tulane Cancer Center to develop and validate drugs for breast cancer therapy.** The Drug Design Team at Xavier consists of experts in computer-aided drug design methods and synthesis and has formed a productive partnership with the Cancer Drug Validation Team at the Tulane Cancer Center. This inter-university collaboration involves training Xavier researchers, including undergraduate students, to carryout the experiments necessary to determine if new compounds would be suitable as new drugs to treat breast cancer. Three separate studies are ongoing as subprojects: (1) Design and synthesis of novel ceramide analogs that potentially reverse chemotherapy drug resistance, (2) Design and synthesis of small molecule inhibitors of HER2 tyrosine kinase to suppress tumorigenesis, and (3) Identification of compounds with the potential for estrogen receptor activity.

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INTRODUCTION:

This project brings together the skills and resources of Xavier University and Tulane University researchers to form a collaborative team in the areas of drug design and validation. This inter-university collaboration whereby a number of *in vitro* and *in vivo* approaches will be applied for validation of lead compounds designed and synthesized at Xavier University will involve training of Xavier researchers and students in drug target validation, biological assays of drug efficacy, evaluation of resistance pathways, and identification of synergistic drug combinations. At Xavier University, Dr. Thomas Wiese is an expert in computer-aided drug design methods, Dr. Maryam Foroozesh is an expert in the design and synthesis of small biologically active organic compounds, and Dr. Jayalakshmi Sridhar is an expert in both fields. On the Tulane Cancer Center side, Dr. Frank Jones is an expert in HER2 positive breast cancers and has an active drug validation program, and Drs. Barbara Beckman and Matthew Burow are experts in the areas of endocrine- and chemo-resistant breast cancers. The distinct contributions of each institution places this collaboration in a unique position to successfully design and validate drugs to address the most pressing challenges in breast cancer therapy. The specific aims of this collaborative project are to develop, promote, and sustain independent, competitive breast cancer research at Xavier University of Louisiana while developing a true partnership between the two institutions.

BODY:

<u>Foroozesh/Beckman/Burow Subproject (Novel Ceramide Analogs as Anti-Cancer Agents)</u>

The research accomplishments of this subproject include the following syntheses and bioassays:

Task 1- Hire research associate to assist in project. (Month 1)

Dr. Jiawang Liu has been working on the DoD project since the beginning of this grant project. He is an expert in the design, synthesis, and bioassays of biologically active molecules.

Task 2- Identify student(s) to assist in project. (Months 1-3)

Over the past two years, a number of Xavier Undergraduate students have been involved in this project.

Task 3- Design and synthesize new ceramide mimicking agents in order to perform structure-activity studies of these novel compounds in hopes of determining important and essential structural features leading to enhanced apoptosis induction. (Months 1-48)

Year 1: The synthesis of ceramide analogs (401, 402, 403, 404 and 406) with different backbones were achieved based on the previously published methods and reported in the Year 1 Progress Report.[1, 2] These specific analogs were designed to help us identify the contributions of the ceramide backbone in the anti-cancer activities. The synthetic scheme for this group of analogs is shown in Figure 1.
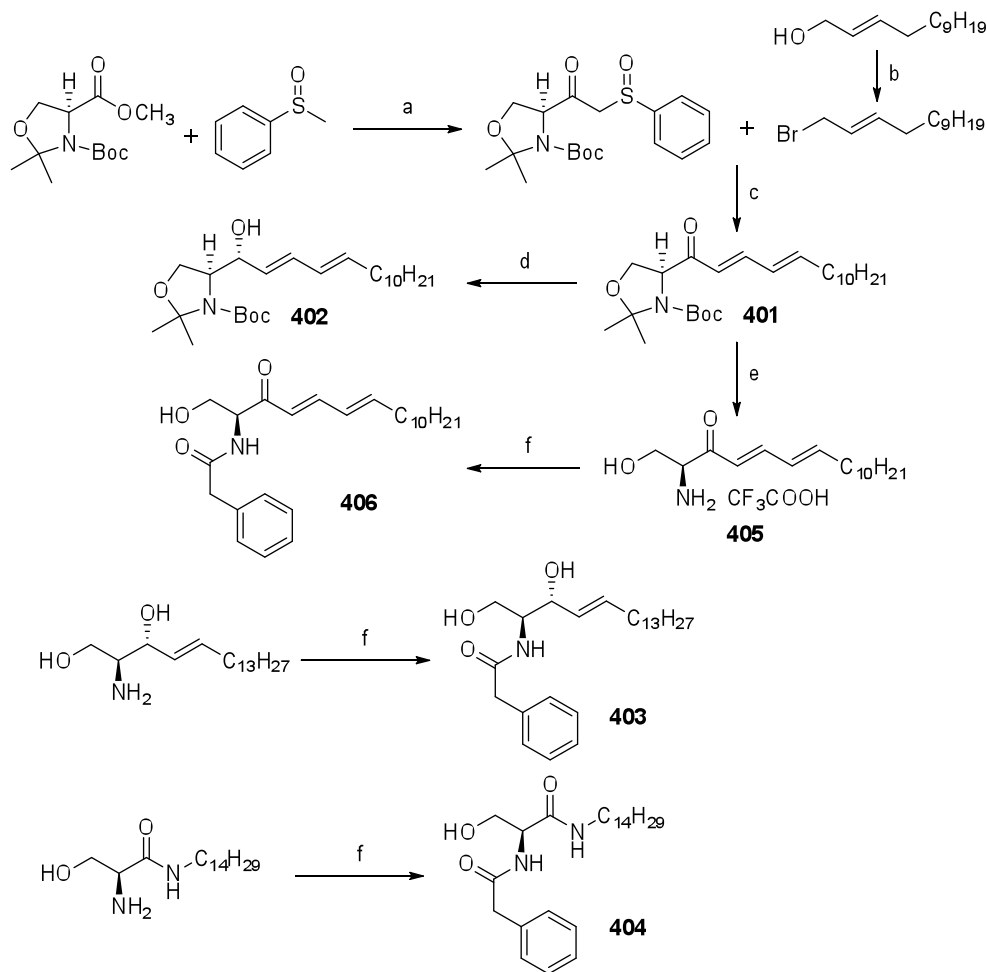

Figure 1. Synthesis scheme for analogs 401-406

Ten ceramide analogs containing the imine group (409, 410, 412, 413, 415, 416, 417, 3T1, 3T2, and 3T3) were also prepared through a facile reaction.[3] Since one of our previously synthesized ceramide analogs containing an imine group has shown the most potent anti-cancer activities,[1] the modifications here are expected to increase the anti-cancer activity and/or provide us with important structure-activity relationship information. The structures of these compounds are shown in Figure 2.
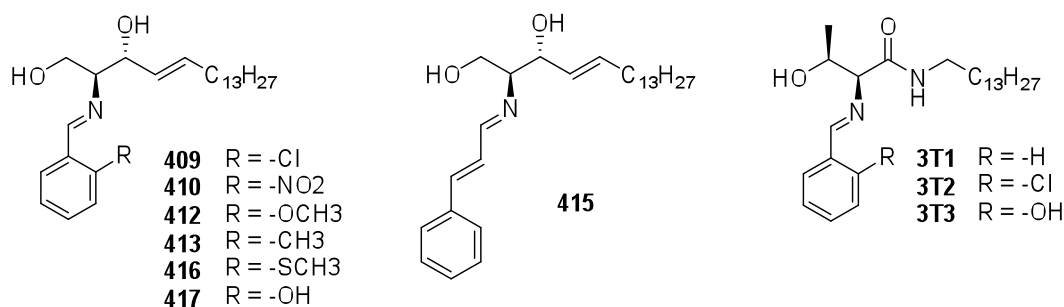
Figure 2. Structures of ceramide analogs 409, 410, 412, 413, 415, 416, 417, 3T1, 3T2, and 3T3

A ceramide analog with the1-hydoxy group blocked (503) was prepared using relatively inexpensive staring materials and simple synthetic route as shown in Figure 3. Compared with the previous synthetic methods,[2] our new synthetic route avoids the usage of expensive starting material methyl (S)-(-)-3-Boc-2,2-dimethyl-4-oxazolidinecarboxylate and excessive protecting and de-protecting steps. Using the starting material O-benzyl-serine, the 1-position blocked ceramide analog (503) was synthesized through a facile four-step route shown below.
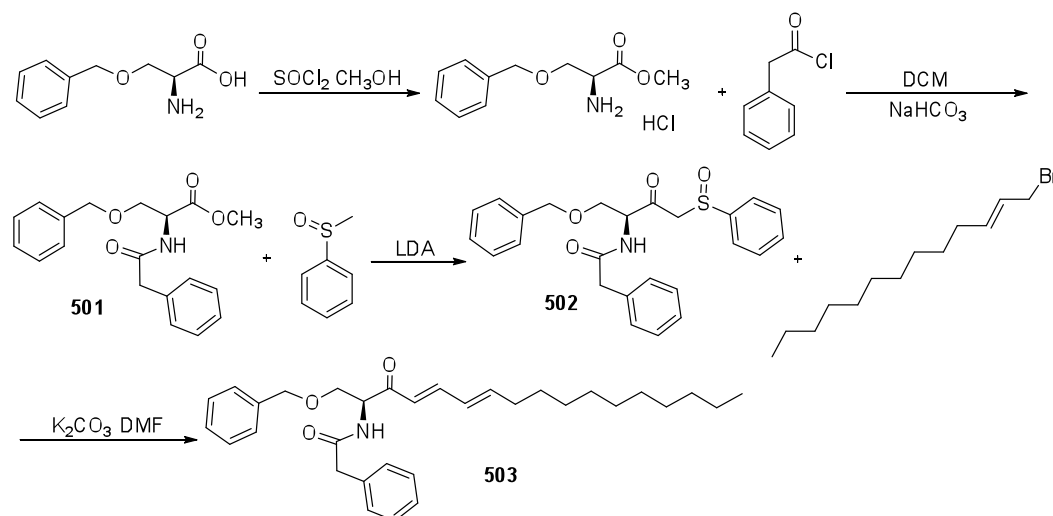

Figure 3. The four-step synthetic route used for the synthesis of analog 503

Year 2: In Year 2, we focused on the synthesis of fluorescent building blocks for incorporation into the new ceramide analogs. Evidence shows that rigid modification of ceramide backbone enhances pro-apoptotic efficacy.[4,5] Employing flavone and coumarin cores as rigid moieties, the long conjugated system-modified ceramide analogs were designed. The synthetic route used is presented in Figure 4. Incorporation of the long conjugated system adds molecular rigidity and fluorescence to the ceramide, facilitating the determination and tracking of the ceramide analogs in bioassays.
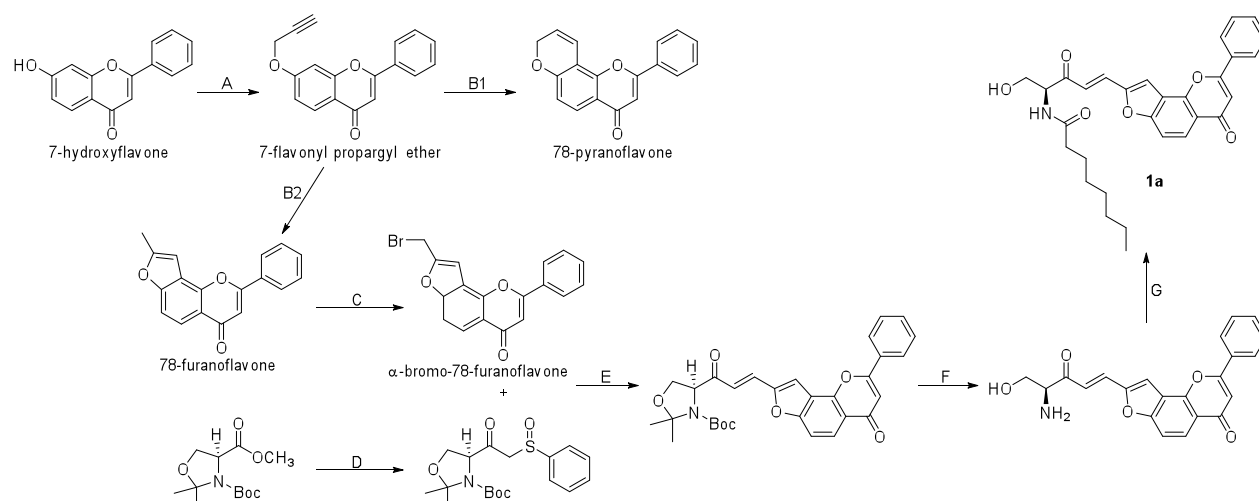
Figure 4. Synthetic route for flavonyl-modified ceramide analogs. Step A) propargyl bromide, potassium carbonate/acetone; Step B1) 200°C in N,N-diethylaniline; Step B2) 200°C cesium fluoride in N,N-diethylaniline; Step C) liquid bromine, aluminum chloride/dichloromethane; Step D) methyl phenyl sulfoxide, lithium diisopropylamine /tetrahydrofuran; Step E) potassium carbonate/N,N-dimethylformamide; Step F) trifluoroacetic acid/dichloromethane; and Step G) octanoic acid, DCC, HOBT/tetrahydrofuran

In Year 2, we successfully synthesized 11 pyranoflavone and 4 furano flavone/coumarin building blocks for the preparation of fluorescent ceramide analogs (Figure 5). Through altering the reaction condition of Claisen rearrangement, pyrano- and furano- derivatives were obtained in a facile method and good yields.
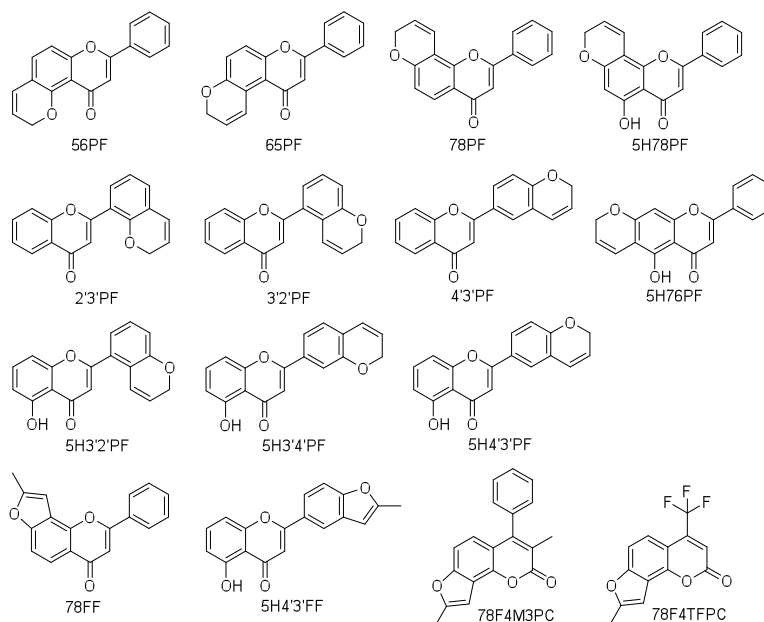

Figure 5. The molecular structures of pyranoflavones, furanoflavones, and furanocoumarins synthesized

In Year 2, we also focused on the determination of molecular conformation of oxazolidine ceramide analogs. Oxazolidine ceramide analogs have shown high anticancer activity in previous studies. Mechanism investigations show that they could induce apoptosis as well as inhibit sphingosine kinase, a ceramide-metabolizing enzyme.[6] Because of the cyclization of 1- position OH group and 3-position amino group, the oxazolidine ceramide analogs possess considerable rigidity and fixed conformations, which are useful for investigating their interactions with molecular targets. Ceramide analogs, 401 and 402 are oxazolidine ceramide analogs. Thus, a conformational investigation of these compounds was carried out in Year 2. 1D NMR spectra show the two sets of signals for each compound. 2D NMR spectra clarify the stereochemistry of compounds. Through molecular simulation and conformational analysis, dual-conformation model of oxazolidine ceramide analogs is generated as seen in Figure 6.
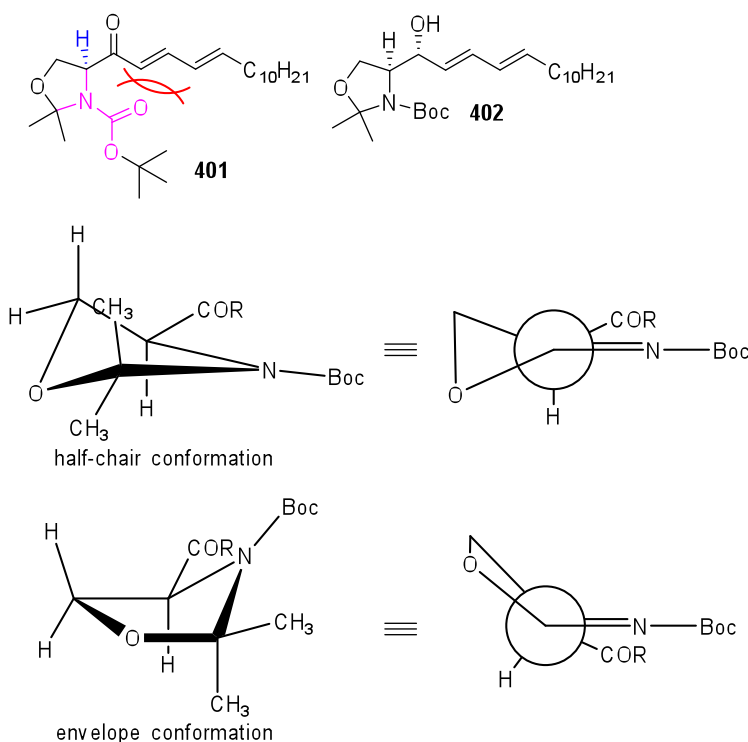

Figure 6. Hypothesized conformations for the conformational isomers of analogs 401. The bulky group $-COCH=CHCH=CHC_{10}H_{21}$ is located above the amide bond plane in the half-chair form, while below the amide plane in the envelope form. In both conformations α-H is in an axial position.

The sp^2 hybridization of N in the amide group results in a planar amide functional group (pink residue). Due to the presence of a bulky group ($-COCH=CHCH=CHC_{10}H_{21}$) on the carbon next to the N in the five-membered oxazoline ring, steric effects lead to the $-COCH=CHCH=CHC_{10}H_{21}$ group locating in the space above or below of the amide

bond plane. Since these two poses cannot freely interchange, two relatively stable conformations are formed which are reflected by the two sets of signals in the NMR spectra. Figure 6 shows the two possible conformations of 401 in half-chair and envelope forms, respectively. In both conformations the α-H (in blue) is axial, which is consistent with the observations in ^{1}H NMR spectra. This new finding, disclosing the conformational isomerism phenomenon of oxazolidine

Task 4- Determination of Anti-Cancer Activities of the Ceramide Analogs. (Months 3-60)

The anti-cancer activities of ceramide analogs 401, 402, 403, 404, and 406 were tested using a cellular viability assay and a clonogenic survival assay in MCF-7, MDA-MB-231, and MCF-7TN-R cells. Compounds 401 and 406 were the most effective compounds across all cell lines with IC_{50} values of 4.05 ± 1.3 µM (p<0.001) and 4.26 ± 1.48 µM (p<0.001) respectively, in the chemo-resistant MCF-7TN-R cell line. Interestingly, IC_{50} values for all analogs except analog 401 were lower in the chemo-resistant MCF-7TN-R and hormone therapy-resistant MDA-MB-231 cell lines, indicating that these compounds exhibit increased therapeutic potential in drug-resistant cancers (Table 1). The fact that two compounds with the 3-ketone-4,6-diene backbone (406 and 401) have shown the most potent anti-cancer activities in this group suggests that the 3-hydroxy-4-ene backbone is not necessary for bioactivity of ceramides as previously believed.[4] The raw results (Figures 7 and 8) are shown bellow.

Our results in apoptosis assays show that analog 406 induces a 4.3 ± 1.1 fold (p<0.05) increase over control in the induction of apoptosis, compared to C8-Cer with a 2.34 ± 0.79 fold increase. Analog 406-induced cell death is mediated through the intrinsic apoptotic pathways, with 3.59 ± 0.45 (p<0.05) fold increase in caspase-9 activity following treatment with the analog. In conjunction with our previous studies, these results suggest that development of ceramide analogs with a diene component in the sphingosine backbone may be well suited for the treatment of chemo-resistant breast cancer.

Table 1. IC$_{50}$ values of ceramide analogs in the MTT viability assay and the clonogenic survival assay (µM). The values are the means of three independent experiments.

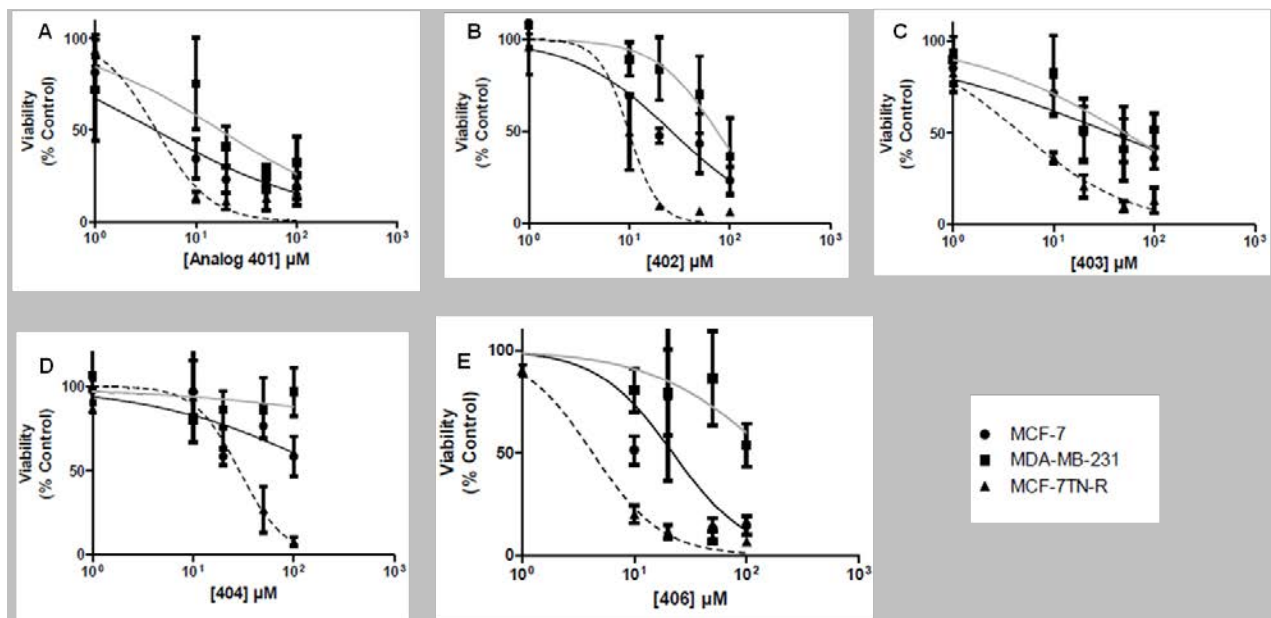|	IC$_{50}$ values in viability assay (µM)			IC$_{50}$ values in survival assay (µM)		
	MCF-7	MCF-7-NTR	MDA-MB-231	MCF-7	MCF-7-NTR	MDA-MB-231
401	3.906	4.047	26.76	5.07	1.854	1.454
402	26.44	9.908	45.54	5.692	5.185	3.174
403	37.28	4.742	35.79	4.175	5.62	1.585
404	233.6	28.96	NE	10.16	10.05	17.55
406	22.03	4.263	81.94	3.403	1.808	1.402

Figure 7. Effect of ceramide analogs on breast cancer viability. MCF-7**,** MDA-MB-231, and MCF-7TN-R cells were treated with increasing concentrations of analogs for 24h. The values are the mean ±SE of three independent experiments.
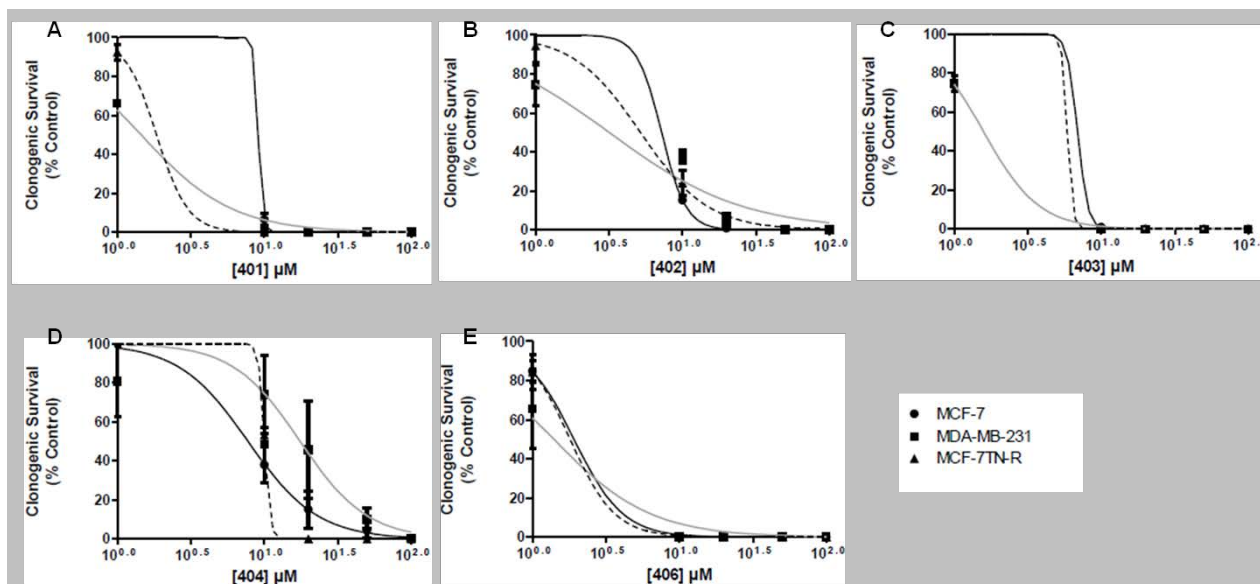
Figure 8. Effect of ceramide analogs on breast cancer clonogenic survival. MCF-7, MDA-MB-231, and MCF-7TN-R cells were treated with increasing concentrations of analogs and allowed to grow until colony formation was noted (generally 10-12 days). The values are the mean ± SE of three independent experiments.

A longitudinal activity comparison of analogs 315, 406, 415 and 503 was performed. These cell viability assays were performed on NCI/ADR-RES, NCI/ADR, OVCAR8, MCF-7, and MCF-7/Dox cells. The results showed that among these analogs, compounds 406 and 415 show the most potent activities. The raw data is provided bellow in Figure 9.

Glucosylceramide synthase (GCS) inhibition assays showed that analog 406 has a mild or no GCS inhibition activity in OVCAR8, NCI/ADR-RES, and NCI/ADR cells. This observation suggests that cytotoxicity of analog 406 is not a result of the inhibition of GCS enzyme. On the other hand, analog 503 showed a significant GCS inhibition activity in all of the tested cell lines. This observation confirms our hypothesis that GCS activity can be inhibited through modification of ceramide's 1-postion. These results provide us with a great perspective for designing novel inhibitors of GCS, an enzyme considered to be critical in cancer drug-resistance. The results of the GCS activity assays are shown bellow in Figure 10.
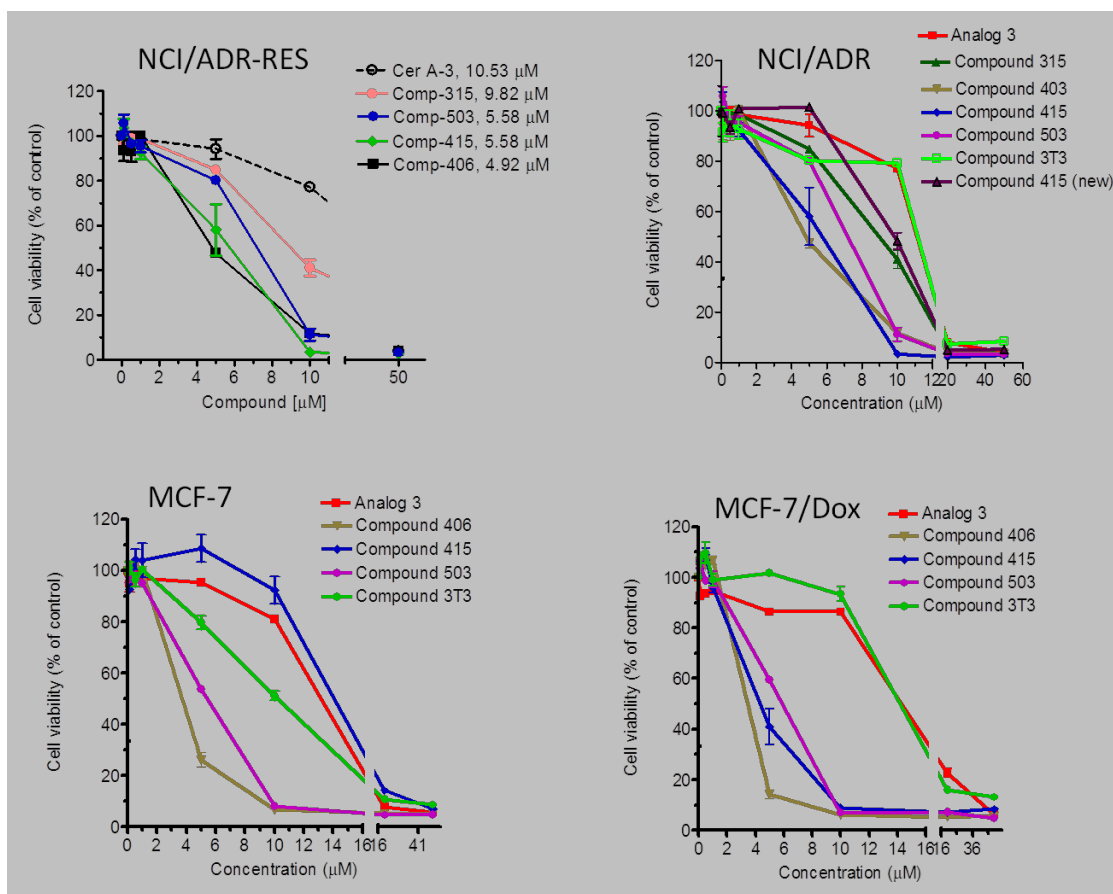
Figure 9. Effect of ceramide analogs on cancer viability. NCI/ADR-RES, NCI/ADR, MCF-7, and MCF-7/Dox cells were treated with increasing concentrations of analogs for 72 h. Analyzed by CellTiter-Glo luminescent cell viability assay from the Promega.

Figure 10. Results of GCS inhibition activity assays in OVCAR8, NCI/ADR-RES, and NCI/ADR cells demonstrated by thin layer chromatograph (TLC). Treatments for 48 hours in 5% FBS RPMI-1640 medium; analyzed by fluorescence enzymatic assay (Gupta V et al *J Lipid Res* 2010, 51:866-74), three times.

<u>Wiese/Burow Subproject (Identification of novel estrogens and antiestrogens in the USDA Phytochemical and FDA Marketed Drugs databases)</u>
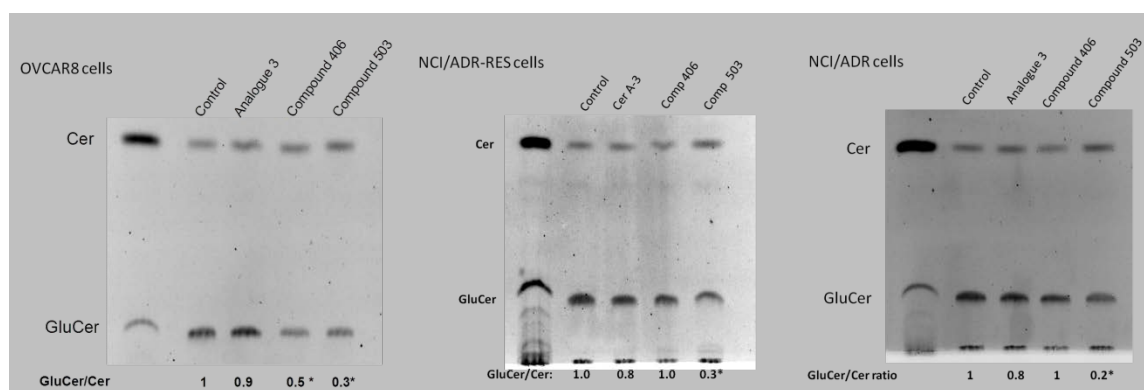
Research accomplishments of this subproject include the following tasks in Specific Aim 1:

Develop structure-based pharmacophore models and ligand-receptor (docking) models for estrogens based on the crystal structures of ER alpha and beta (with bound agonists or antagonists) and then virtually screen the USDA Phytochemical, Chinese Herbal Medicine, and the FDA Marketed Drug Databases for new estrogens.

Task 1- Identify student to assist in project. (Month 1)

Year 1: Pharmacy students Chioma Obih and Felicia Gibson who have worked in the Wiese lab for the previous 2 years were assigned to this project in Fall 2011. Both students are supported by the College of Pharmacy Center of Excellence Grant. Dr. Wiese trained Ms. Obih on structure-based modeling methods using the MOE software and she worked with Dr. Wiese on Task 2. Ms. Gibson focused on working with Ms. Candace Hopgood on *in vitro* bioassays.

Year 2: Ms. Obih graduated in Spring 2013 and left the group. Starting January 2013, Ms. Gabriela Barbarini, a pharmacy exchange student from Brazil started working in the Wiese lab learning bioassay techniques used in Task 6. Ms. Barbarini is a third year pharmacy student doing one year in the US at Xavier's college of pharmacy and selected to work in the Wiese lab. During summer of 2013, Ms. Barbarini worked in the laboratory of Dr. Burow at Tulane (DOD Project Collaborator of Dr. Wiese) and learned additional bioassay methods. She is now back in the Wiese lab, applying her skills to evaluate the estrogen activity of compounds identified in this project in a 3 credit research experience course.

Task 2- Develop structure-based pharmacophore models for estrogens. (Months 1-4)

2a - Obtain all crystal structures of ER LBDs (Month 1)

Year 1: A search of the Protein Database in Fall 2011 resulted in the identification of 62 crystal structures of the human Estrogen Receptor (ER) ligand-binding domain (LBD), all of which contained one bound ligand. These LBD structures were processed and aligned relative to each other so that similarities and differences in ligand-binding pockets could be identified.

2b - Sort LBD structures by cavity shape and helix-12 position (Months 1-3)

In preliminary studies prior to this project, we have shown that ligand receptor docking (or virtual screening using docking) can produce very different results between ER LBD structures containing steroid or stilbene ligands, even though both ligands are agonists and the LBD cavity sizes are very similar. The Xavier Molecular Structure and Modeling Core was utilized to compare the ligand-binding cavity sizes of the 62 processed structures. At the same time, a manual sorting was undertaken to group ER LBD crystal structures by bound ligand type, cavity size, and position of helix 12. This process resulted in the identification of 26 structures in the antagonist configuration (helix 12 open) and 36 structures in the agonist configuration with helix 12 closed. While cavity volume did not clearly group these structures, a combination of cavity size and bound ligand type was used to select representative agonist and antagonist crystal structures of the ER LBD. These 5 agonist structures (1ERE, 2G50, 2P15, 2QH6, and 3ERD) and 3 antagonist structures (1ERR, 3DT3, and 3ERT) will be used in the structure-based database screening.

Year 2: A computational mythology was identified that can utilize all of the ER LBD structures for the virtual screening rather than a subset of 5. The new strategy is to use as many of the 62 ER LBD structures as needed for docking the target databases based on the similarity of each database compound to the bound ligands of each available ER LBD. The critical part of this method involves sorting the databases by similarity to the bound ligands of all 62 ER LBD structures. Then, database members most similar to any bound ligand are docked into the corresponding ER LBD crystal structure. This approach should reduce false positives and negatives in the virtual screening since the ER LBD is known to take on slightly different ligand binding cavity shapes as it binds to different ligands. In order to apply this methodology to this project, Dr. Wiese attended a one-day training workshop held by Chemical Computing Group (Makers of MOE software) in May 2013. From this training, a process was developed to presort the databases before docking and the skills were obtained to carry out the process. This new workflow for the screening and validation process has been refined to the following:

1. Wash and Filter Databases to molecules that may bind to ER LBD (same as original plan).

2. Sort databases into subsets by similarity to the ligands bound to the 62 ER LBD structures.

3. Dock sorted database subsets into corresponding ER LBD that have been aligned by binding cavity.

4. Repeat steps 2 and 3 after Meteor-derived Phase I metabolites are generated.

5. Select compounds for validation with bioassays based on docking scores.

This process is taking longer than anticipated to do the sorting and is currently at steps 2 and 3.

2c - Develop pharmacophore models from representative LBD structures (Months 1-4)

Since all of the ligands in the selected ER LBD structures bind to the ER using similar interactions, the development of classical pharmacophore models for the ER LBD models was determined to be unnecessary. Structure-based screening for this project will utilize docking to the selected crystal structures where typical pharmacophore interactions are part of the ligand pose generation and score process.

Task 3- Mine phytochemical and marketed drug databases with pharmacophore models. (Months 3-5)

3a - Evaluate Docking methods for virtual screening of estrogens (Months 4-8)

Year 1: The Xavier Molecular Structure and Modeling Core was utilized to quickly evaluate the potential for the MOE, Gold, Glide, and Surflex Dock to be used for docking into the ER LBD. FlexX is no longer used in this lab, and the Glide method is an additional method used by the Molecular Structure and Modeling Core. This fast study using default setting of the software to replace the bound ligand into the binding cavity did not identify significant differences in the performance of these methods. Considering that the Wiese lab has the most experience with the MOE software and the fact that the MOE software is the only docking package available to us that can include a force field optimization, MOE was selected for further optimization studies.

Year 2: Each ER LBD structure was evaluated and processed for docking using the MOE Structure Preparation Module.

3b - Ligand replacement optimization docking of representative LBD structures using MOE, Gold, FlexX, Surflex Dock (Months 4-7)

Year 1: A systematic ligand replacement study was performed using the MOE software, and an optimal configuration of the MOE docking method was identified that produced ligand replacement very close to the crystal structure (low RMSD). The MOE docking software, with its multiple ligand placement and scoring methods, was systematically evaluated for ability to replace the bound ligand in representative examples of the ER agonist and antagonist LBD structures.

Year 2: A method using the Triangle Matches Placement Method and the London dG scoring followed by a ligand minimization and final rescoring with method GBVI/WSA dG was found to produce ligand replacements very close to the crystal structure.

Task 4- Refine pharmacophore selection of estrogens using docking. (Months 6-8)

Year 1: Not initiated.

Year 2: The phytochemical (76,451 compounds) and marketed drug databases (16,096 compounds) were obtained from the Xavier Molecular Structure and Modeling Core in SDF format. The process of processing these databases for virtual screening is currently underway. This process includes creating all tautomers, isomers, enantiomers, and filtering out compounds too large to bind ER using MOE. In addition, the software Meteor will be used to create potential Phase I metabolites of each structure.

The process of sorting these databases by similarity to the bound ligands of the 62 ER LBDs is also underway (see Task 2b above).

This process has been also started on test database subsets.

Task 5- Hire research associate to assist with *in vitro* assays. (Month 7)

Year 1: Ms. Candace Hopgood has worked in the Wiese lab for 2 years and was transferred to this project briefly in spring and summer of 2012. She spent the summer testing some of the bioassays to be used in the validation phase of this project including the Lantha Screen ER binding assay and the labs MVLN and T47D reporter gene assays. Ms. Hopgood left the Wiese lab in Fall 2012 to focus on her application to Xavier Pharmacy School where she is now a P1 student.

Year 2: In January 2013, Ms. Peng Ma was partially reassigned to the bioassay component of the project (50% effort). Ms. Ma also continues to work with Dr. Wiese in the RCMI (NIH-funded Research Centers in Minority Institutions) Cell and Molecular Biology Core (at 50% effort) that he directs. Ms. Ma is very skilled with most of the *in vitro* methods used to validate the virtual screening in this project. She also is training and working with Ms. Barbarini (a new student) in the lab.

Task 6- *In vitro* validation of estrogen activity. (Months 8-30)

6a - Obtain samples of 10-20 test compounds selected in virtual screen (Months 8-9)

Year 1: Not initiated.

Year 2: A preliminary database of 29 stilbene analogs has been obtained from the USDA Natural Products Utilization Research Unit in Mississippi. The stilbene structure core has been used as the basis for potent ER agonists and antagonists that are in the registered pharmaceuticals and herbal medicine databases. The 29 analogs obtained have been characterized for anticancer effects, but not evaluated for estrogen activity.

6b - Perform FP ER-alpha/beta binding determinations of test compounds (Months 9-15)
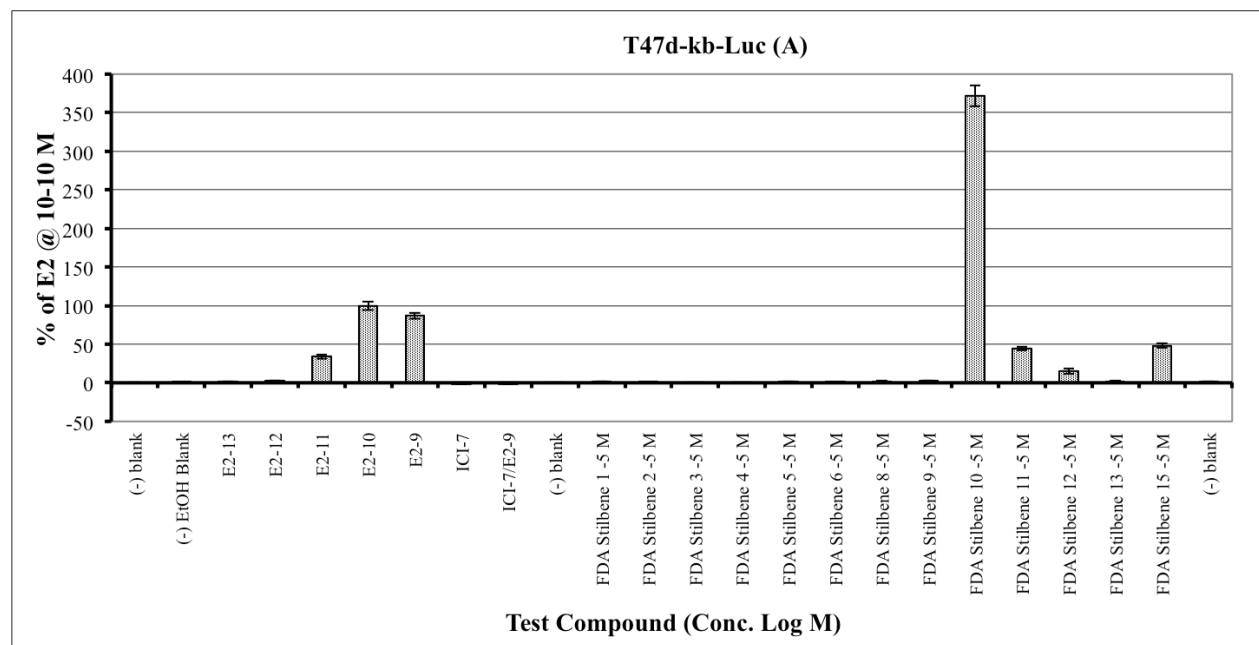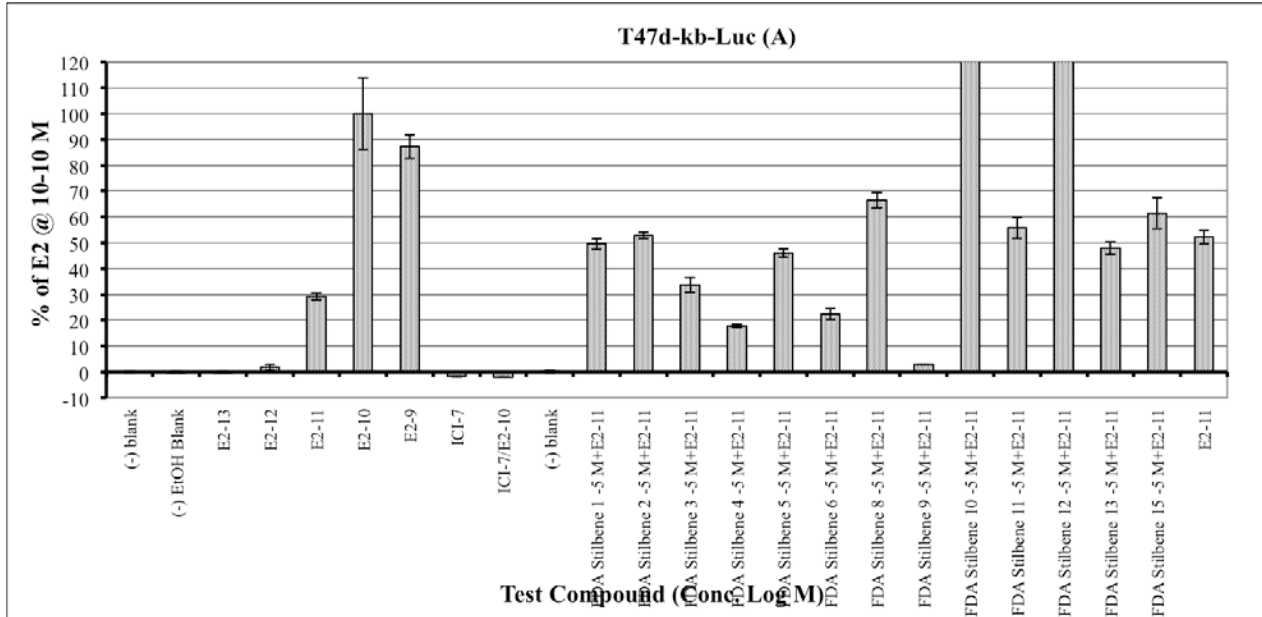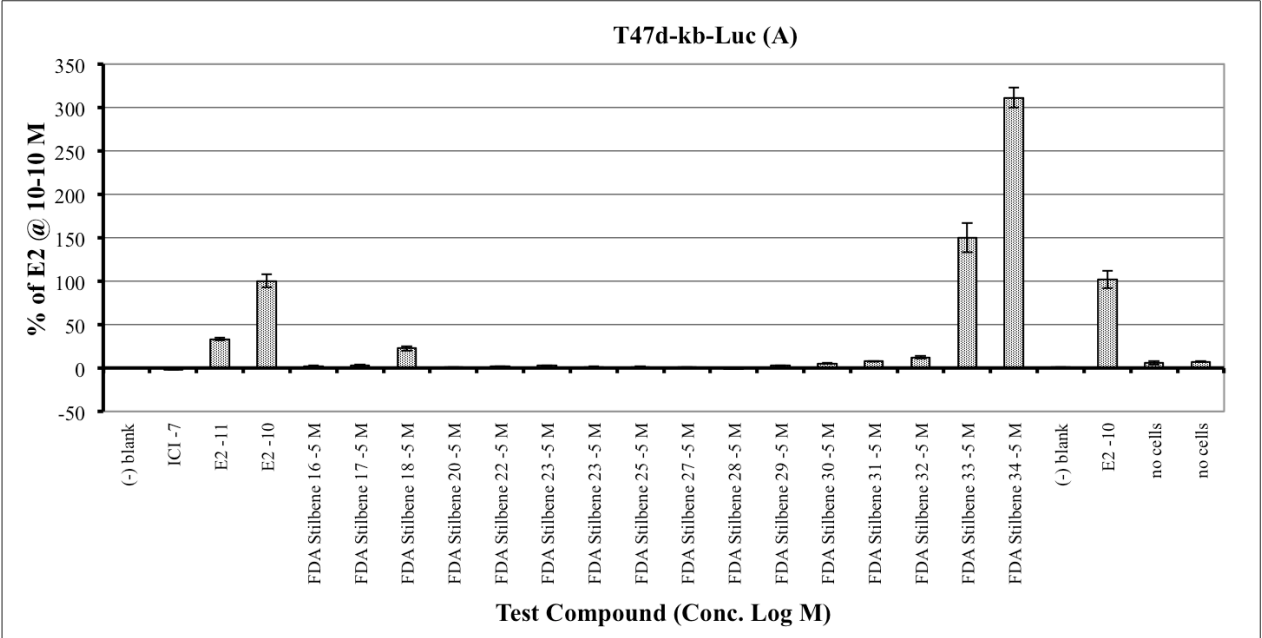
Year 1: Not initiated.

Year 2: Dr. Wiese has been training Ms. Ma to carry out the ER binding assays and she has developed her proficiency to a level that she will soon run ER alpha and beta binding curves for the 29 stilbenes.

6c - Perform MVLN reporter gene agonist/antagonist determinations of test compounds (Months 12-18)

Year 1: Not initiated.

Year 2: The 29 stilbene compounds have been used as a test set to train and standardize the ER responsive reporter gene assays in the Wiese Lab. All compounds were tested in thee experiments at 10 uM in the T47Dkb-Luc cells to check for agonist and/or antagonist activity. Rather than using the MVLN reporter gene cells, the T47Dkb-Luc cells have been used since they provide a stronger estrogen response and since they are 10X more sensitive to estrogens. Representative data are shown below. Five stilbenes were found to be ER agonists, 3 exhibit antagonist activity and 7 stilbenes potentiate the activity of estradiol.

T47d-kb-Luc (A)

% of E2 @ 10-10 M

350
300
250
200
150
100
50
0
-50

(-) blank
ICl -7
E2 -11
E2 -10
FDA Stilbene 16 -5 M
FDA Stilbene 17 -5 M
FDA Stilbene 18 -5 M
FDA Stilbene 20 -5 M
FDA Stilbene 22 -5 M
FDA Stilbene 23 -5 M
FDA Stilbene 23 -5 M
FDA Stilbene 25 -5 M
FDA Stilbene 27 -5 M
FDA Stilbene 28 -5 M
FDA Stilbene 29 -5 M
FDA Stilbene 30 -5 M
FDA Stilbene 31 -5 M
FDA Stilbene 32 -5 M
FDA Stilbene 33 -5 M
FDA Stilbene 34 -5 M
(-) blank
E2 -10
no cells
no cells

Test Compound (Conc. Log M)

T47d-kb-Luc (A)

% of E2 @ 10-10 M

120
110
100
90
80
70
60
50
40
30
20
10
0
-10

(-) blank
(-) EtOH Blank
E2 -13
E2 -12
E2 -11
E2 -10
E2 -9
ICl -7
ICl-7/E2 -10
(-) blank
FDA Stilbene 1 -5 M+E2 -11
FDA Stilbene 2 -5 M+E2 -11
FDA Stilbene 3 -5 M+E2 -11
FDA Stilbene 4 -5 M+E2 -11
FDA Stilbene 5 -5 M+E2 -11
FDA Stilbene 6 -5 M+E2 -11
FDA Stilbene 8 -5 M+E2 -11
FDA Stilbene 9 -5 M+E2 -11
FDA Stilbene 10 -5 M+E2 -11
FDA Stilbene 11 -5 M+E2 -11
FDA Stilbene 12 -5 M+E2 -11
FDA Stilbene 13 -5 M+E2 -11
FDA Stilbene 15 -5 M+E2 -11
E2 -11

Test Compound (Conc. Log M)

6d - Perform ER alpha/beta selective reporter gene assays of test compounds (Months 12-20)

6e - Evaluate test compounds in estrogen/breast cancer PCR array (Months 16-30)

6f - Data analysis of PCR array data (Months 28-33)

6g - Evaluate coactivator ER interactions with test compounds bound using LanthaScreen™ TR-FRET ER alpha/beta Coactivator Assay (Months 16-35)

6h - Perform genome wide shRNA library screen coupled with gene expression arrays of sensitive cells to identify drug targets, drug sensitizers, and drug-resistance pathways (Months 18-30)

Tasks 6d-6h have not been initiated.

Task 7 - *In vivo* validation of estrogen activity. (Months 33-48)

7a - Test compounds for uterotrophic activity in mice (months 33-37)

7b - Examine antiestrogenic capacity of test compounds in uterotrophic assays (months 34-38).

7c - Evaluate activity of test compounds on breast cancer xenografts (months 38-48).

Task 7 has not been initiated.

Sridhar/Jones/Stevens Subproject (Identification of a New Class of Tyrosine Kinase Inhibitors)

The research accomplishments of this subproject include the following:

Task 1- Hire research associate to assist in project. (Month 1)

Dr. Jayalakshmi was hired as a research associate. Her expertise in organic chemistry and skills in molecular modeling made her an ideal fit for this project. In August of 2012, she joined the Xavier University Chemistry Department in a new position as a tenure-track Assistant Professor. In her new capacity, she co-directs this subproject with Dr. Cheryl Stevens who has left Xavier for a position as the Dean of the College of Science and Engineering at Western Kentucky University. Dr. Stevens and Dr. Sridhar have agreed to continue collaborating on this subproject with the goal of developing Dr. Sridhar into a prolific and well-trained cancer researcher.

Task 2- Identify student to assist in project. (Month 3)

Year 1: Due to Dr. Stevens leaving Xavier University in January 2012, no students were hired on this project in Year 1.

Year 2: Two students have been working on the project in Year 2 (Thuy-Linh Nguyen, and Jasmine Thompson).

Task 3- Identify novel small molecules related to quinazoline, tyrphostin, emodin, and dasatinib that inhibit HER2 activity. (Months 1-24)
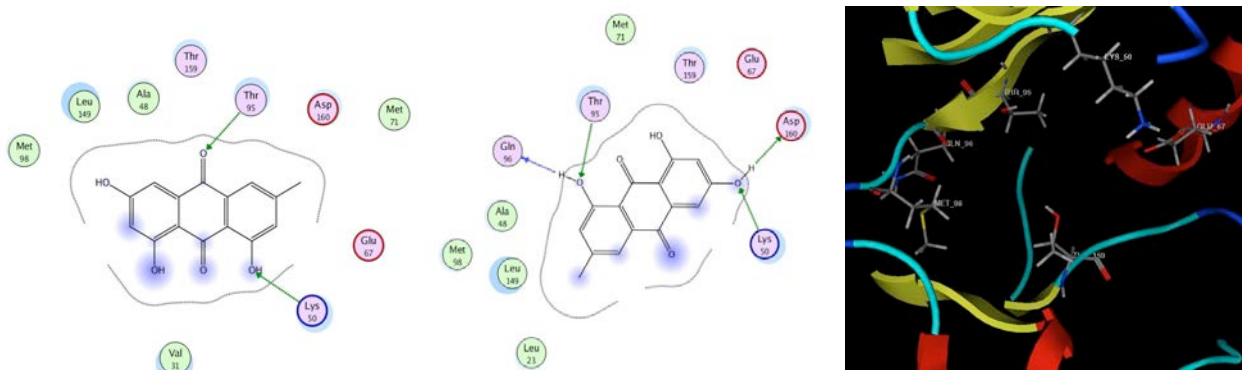
3a - Identify detailed pharmacophore and determine geometric, electronic, and lipophilic characteristics required for tyrosine kinase inhibition (Months 1-12)

HER2 is a growth factor receptor protein belonging to the tyrosine kinase receptor family. HER2 is overexpressed in 25-30% of breast cancer patients and its overexpression has been detected in several other cancers including prostate cancer, ovarian cancer, lung cancer, mammary carcinoma, liver tumors and colorectal cancers. Trastuzumab is a humanized antibody targeting the extracellular domain of HER2 that is currently being used clinically. Among the many tyrosine kinase inhibitors developed so far, only Lapatinib is in clinical use. Several other HER2 kinase inhibitors are in various stages of clinical trials.

The splice variant HER2Δ16 isoform lacking exon 16 preceding the transmembrane domain shows low sensitivity to Trastuzumab. This makes the development of a HER2 kinase inhibitor a more reasonable approach. Castiglioni, et al., (*Endocrine-Related Cancer* (2006) **13**, 221-232) have shown that emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) was the only drug that inhibited the therapeutically

resistant oncogenic HER2 isoform, HER2Δ16. Based on these reports, emodin was chosen as the lead structure for development of HER2Δ16 inhibitors. Emodin and Iressa were first docked onto the HER2 homology model to study their binding modes with the help of MOE docking tools. Iressa did not bind to the hinge region residues of the protein. However, emodin did bind to the hinge region of the protein and three binding modes were identified (Figure 1). Based on the orientation of emodin in the binding pocket of the protein, residues that could be targeted for developing a good inhibitor were identified. These were Thr95, Gln96, Met98, Asp160, Lys50, Glu67, Thr159 (Figure 1).

Figure 1. Binding modes of emodin onto HER2 protein homology model and a picture of the binding pocket with the potential target residues depicted in stick mode.

 3b - Identify new compounds to be tested for tyrosine kinase inhibition with conformationally flexible searches of compound databases using detailed pharmacophore and CoMFA QSAR results. (Months 9-24).

Year 1: 1,3,8-trihydroxyanthraquinone was taken as the pharmacophore for a UNITY 2D-search of all the databases available to us. Hits were obtained from ACD (10 hits), TSCA (3 hits), and NCI databases (39 hits). NCI database hits overlapped significantly with the compounds contained in ACD hits.
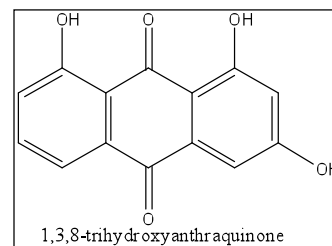

1,3,8-trihydroxyanthraquinone

Table 1: High-throughput screening of database hits against MCF7-HER2Δ16 cell line.

Compound	% of E2 at 10^{-5}M
AG-650/41069241	91.27
AG-650/41069319	11.11
AG-650/41069355	98.16
AP-782/41885488	0.26
AQ-776/42801622	87.79
AE-508/36399063	95.19
AP-782/21243033	96.81
AN-967/15488023	95.16
AG-650/41069356	0
AE-848/13198350	100
NSC322354	0.04
NSC227279	0.1
NSC109351	52.35
NSC202069	95.71
NSC299384	74.26
NSC309875	88.56
NSC309876	89.11
NSC310337	82.17
NSC310338	94.35
NSC319437	82.2
NSC367088	88.97
NSC379572	96.6
NSC379866	96.79
NSC93419	18.04
NSC7794	100.94
NSC138557	80.5
NSC204855	9.11
NSC251670	101.55

Year 2: Based on the initial high-throughput assay against MCF7-HER2Δ16 cell lines (Table 1), 13 compounds were chosen for further analysis (Table 2). Structures of these compounds are shown in figure 2. Additionally 3D and 2D-databases are being compiled using the commercially available compounds from TIMTEC, LC laboratories, Maybridge, and Pubchem. Based on the docking studies (explained in Task 4a) of the active compounds 4, 5 and 13 from table 2; the essential features required for the inhibitor pharmacophore has been reduced to 5-hydroxy-2-(hydroxymethyl)naphthalene-1,4-dione. Database searches are currently underway to find new compounds that satisfy this pharmacophore. The hits obtained from the database searches will be docked and common core structures will be identified as new lead molecules.
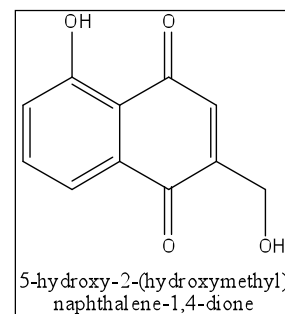

5-hydroxy-2-(hydroxymethyl)naphthalene-1,4-dione

Table 2: The 13 compounds that were chosen for further analysis

No.	Compound
1	NSC313**36**
2	NSC4011**45**
3	NSC2574**50**
4	NSC3223**54**
5	NSC2272**79**
6	AG-650/41069**129**
7	AG-650/41069**131**
8	AG-650/41069**241**
9	AG-650/41069**319**
10	AG-650/41069**356**
11	AG-650/41069**360**
12	AG-650/41069**378**
13	AP-782/41885**488**

Figure 2: Structures of the 13 compounds listed in table 2.
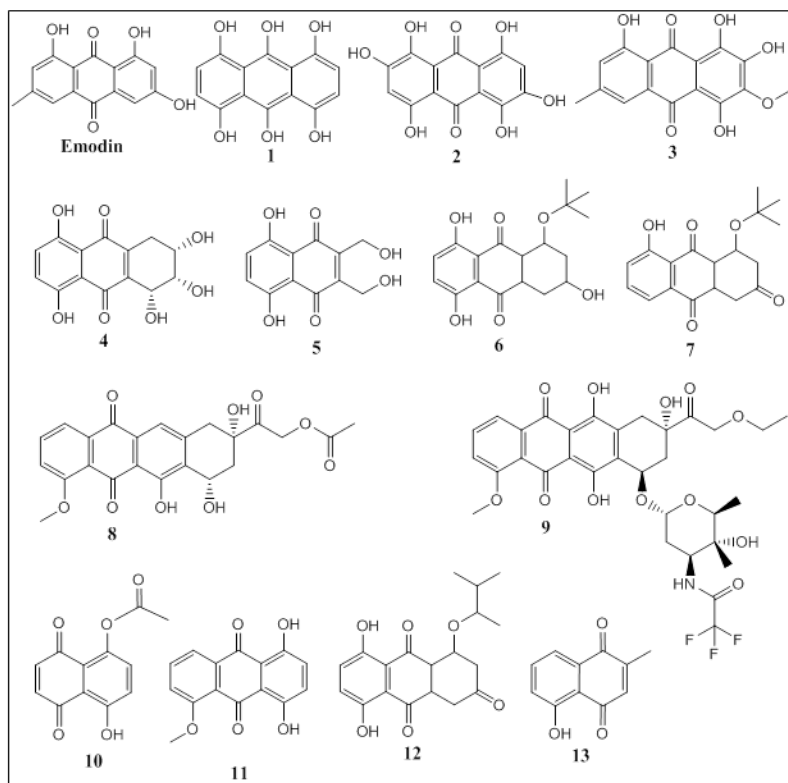
Task 4- Explore the mechanism of HER2 tyrosine kinase inhibition. (Months 12-48)

4a - Dock proven and proposed TKIs into the tyrosine kinase ATP binding site using multiple poses, and score results (Months 12-24)

Year 1: All of the hits described in **3b** were then docked onto the homology model of HER2 using MOE dock tools. The docking results were then studied manually. Binding of the molecule to one of the hinge region residues THR95, GLN96, MET98 was taken as a prerequisite. The number of ligand-protein hydrogen bond interactions, the extent of penetration of ligand into the pocket and the nature of ligand solvent exposure (hydrophobic/hydrophilic) were also considered.

Year 2: The three compounds that showed significant potency against the MCF-7 pcDNA, MCF7-HER2 and MCF-7 HER2Δ16 were subjected to docking studies onto the 3D-structure of HER2 kinase region (PDB ID: 3CRD.pdb) using MOE docking module and Surflex in SYBYL-X1.3. The consensus binding modes for these three compounds are shown in Figure 3. The docking modes of the compounds show that the phenolic group forms a hydrogen bond with the hinge region residue Gln799 and an additional hydrogen bond by the side chain hydroxyl group either with the invariable Lys753 for compound **4** or with Asp863 for compound **5**. Compound **13** made only one hydrogen bond with the protein which could account for its lower potency (refer to bioassay results in Task 4d). This lead us to the optimal core structure of 5-hydroxy-2-(hydroxymethyl)naphthalene-1,4-dione as our lead structure.
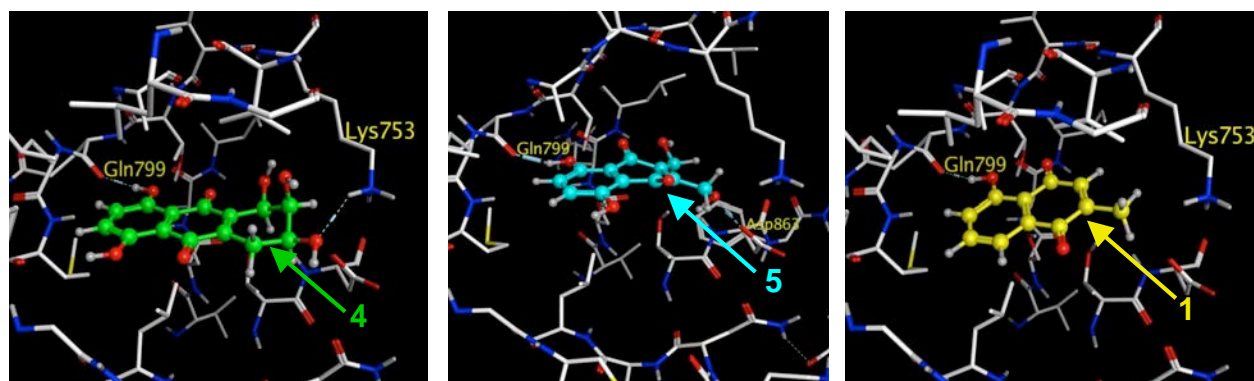

Figure 3: (A) **4**, (B) **5** and (C) **13** in the active site of HER2 kinase. The protein (PDB ID: 3CRD.pdb) residues are shown as stick models and the compounds are shown as ball & stick model.

**4b -** Optimize molecular structures to maximize ability of compounds to inhibit HER2 (Months 15-30)

Year 1: Not initiated.

**Year 2:** From the bioassay results (Task 4d) and the docking studies it was clear that

two core structures that can maintain the two hydrogen bonds shown by compound **5** can serve as the lead molecules **LM1** and **LM2**. The synthetic protocol for the lead molecule LM1 has been designed as given in the scheme 1 involving many known
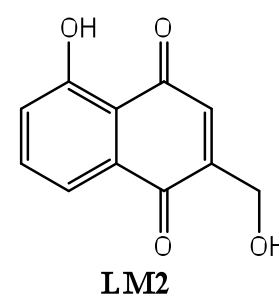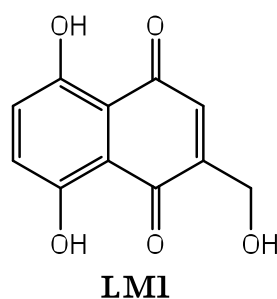
reactions (Tanoue and Terada, *Bull. Chem. Soc. Jpn.,* (1988) **61**, 2039). The reactions until the formation of compound **17** have been standardized. Different Grignard reagents will be used to obtain variation in the R-group on compound **17**. The purification of compound **17** and its conversion to LM1 by oxidation with cerric ammonium nitrate is currently being pursued.
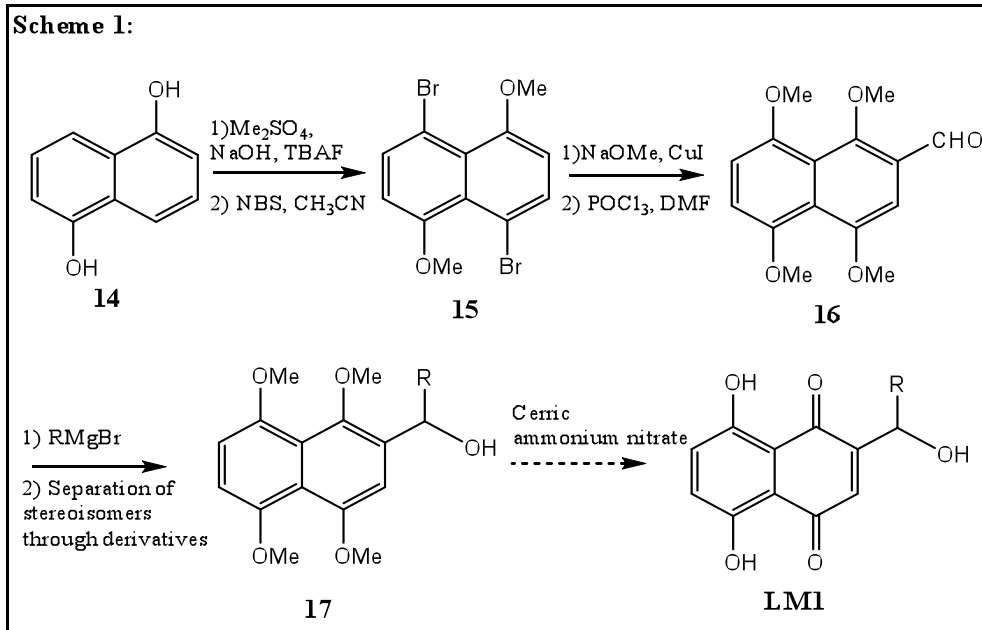
Scheme 1:

Scheme 2 provides an alternate route for the synthesis of the lead compound LM1 by using the Diels-Alder reaction (Paull *et al., J. Med. Chem.,* (1976), **19**, 337). The reactions in this scheme for the formation of the dihydroxynaphthoquinone **18** have been standardized. Further reactions are presently being carried out. Once the final diol is obtained from compound **18**, further reactions will be performed to obtain derivatives with different functional groups on the side chains and with differing chain lengths.
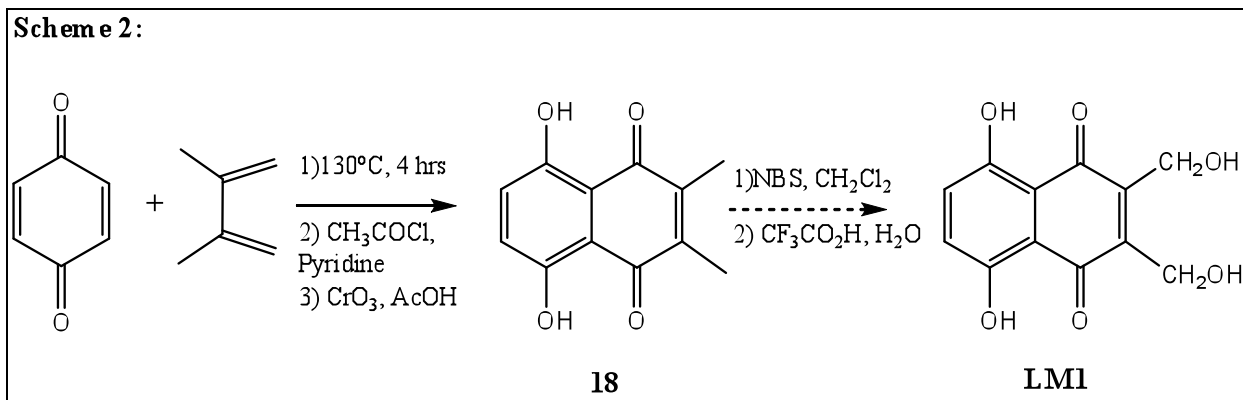
Scheme 2:

The synthesis of the lead molecule **LM2** will be pursued using scheme 3 which is similar to scheme 2 in that both the schemes involve the Diels-Alder reaction as the first step. The first reaction to compound **18** in scheme 3 is presently being standardized. Upon standardization of the reactions, different derivatives of LM2 will be obtained by using suitably substituted starting materials and/or by derivatization of the hydroxynaphthoquinone LM2.

Scheme 3:
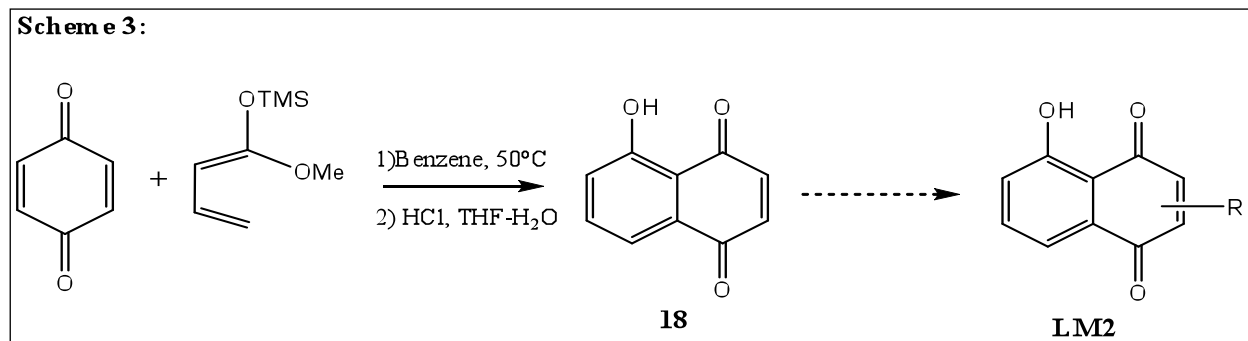
**4c -** Attempt to identify alternate binding sites (Months 18-30)

No progress yet.

4d - Perform *in vitro* kinase inhibition and binding assays (Months 18-48)

Year 1: A total of 28 compounds were procured from the Developmental Therapeutics Program NCI/NIH and Specs chemicals (Table 1). An initial high-throughput assay was performed to determine the inhibition of proliferation of MCF-7 cell line. The compounds that showed good inhibition activity were then subjected to *in-vitro* assay against HER2Δ16 cell line activity. Two compounds that showed low inhibition activity were included to confirm the activity profile of this set of compounds. Two of the tested compounds (NSC322354 and AG-650-41069319) showed low micro molar activity against the HER2Δ16 cell line (< 10 mM) (Table 1).

Year 2: We tested the ability of 13 small molecules (selected based on the high-throughput screening results given in Table 1) that are structurally similar to emodin to inhibit cell viability in MCF-7 breast cancer cell lines that express HER2, HER2Δ16 or empty vector. To do this we treated the cells for 48 hours with a concentration of 10μM for each set of drugs. After treatment, cell viability was tested using the CellTiter-Glo Assay (Promega) (Figure 4A). Of the 13 compounds, we found three that suppressed cell viability potently in all three cell lines (NSC322354 (Drug 4), NSC227279 (Drug 5), and AP-782/41885488 (Drug 13)). After 48 hours, lapatinib decreased cell viability by <50% while the three test drugs decreased cell viability by >90% at the same concentration (Figure 4B).
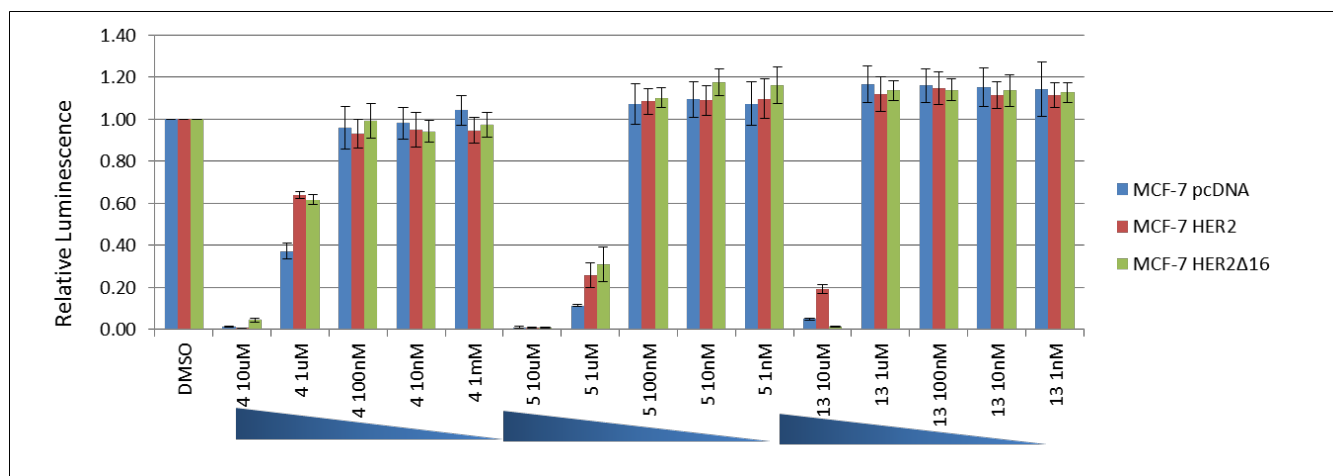
A:
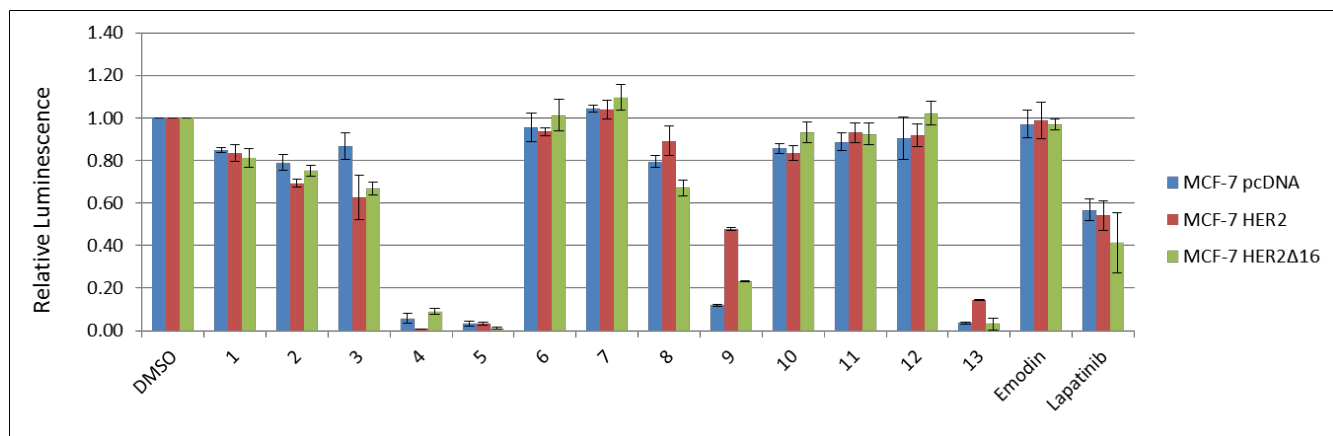
B:

Figure 4: Inhibition of MCF7-pcDNA, MCF7-HER2 and MCF7-HER2Δ16 cell lines (A) High-throughput assay of emodin and compounds 1 – 13, (B) Inhibition assay of compounds **4**, **5** and **13** at different concentrations.

To determine the ability of the drug treatments to inhibit the activation of receptor tyrosine kinase HER2, Western blots were performed to detect total phosphorylated protein after treating each cell line with the three most potent drugs or lapatinib at 10μM for 2 hours. As expected, lapatinib dramatically decreases HER2 activating phosphorylation at auto-phosphorylation site Y1248 respectively in each of the cell lines. These sites were suppressed to the same extent in the cell lines after exposure to each of the test drugs, indicating that, like lapatinib, activation of both receptors was repressed by the test compounds effectively and quickly. We also tested phosphorylation of HER2 after exposure to one of the compounds (AP-650/41069356 (Drug 10)) that we found to have no effect on cell viability. This drug had no effect on

the phosphorylation status of HER2, suggesting that the ability of the drugs to inhibit these receptor tyrosine kinases is critical to their ability to induce cell death.
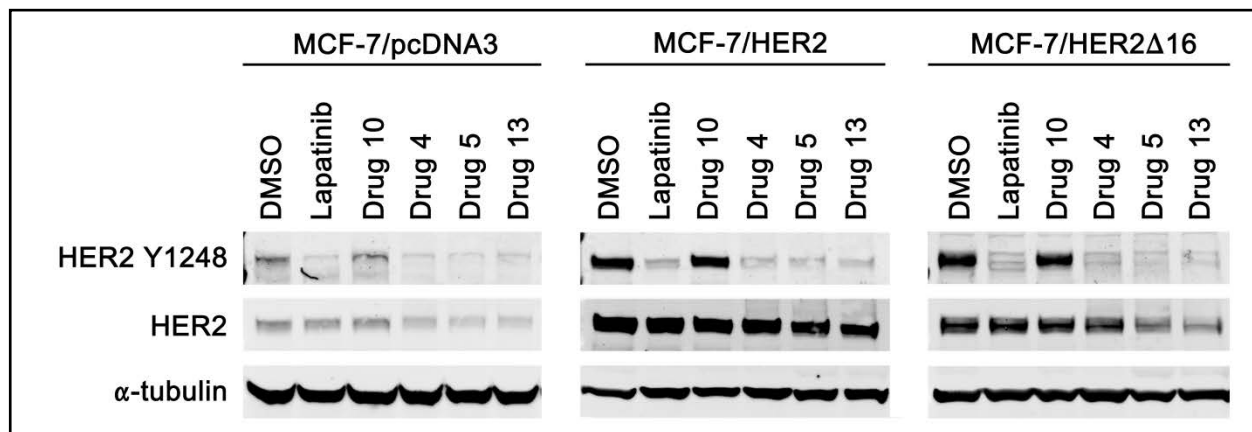

Figure 5: Western blot analysis of autophosphorylation at the HER2 residue Y1248. Compounds **4, 5** and **13** inhibit the phosphorylation at Y1248 in MCF7/pcDNA, MCF/HER2 and MCF7/HER216. Compound 10 which did not show notable inhibition in the high-through put assay shows significant phosphorylation at Y1248 in all of the three cell lines.

Finally, we measured the IC_{50} of each drug by treating each of the cell lines with different drug concentrations for 48 hours then using the CellTiter-Glo Assay to detect cell viability (Figure 6). Cells overexpressing HER2Δ16 showed resistance to lapatinib (IC_{50} = 19.22µM) compared to wildtype HER2 overexpression (IC50 = 15.79µM). All three of the test drugs had a low µM IC_{50} for each of the cell lines. We found that the two most effective drugs at inhibiting cell viability (NSC322354 (Drug 4), NSC227279 (Drug5)) also had similar IC_{50} concentrations for both the HER2 (0.87µM, 0.82µM) and HER2Δ16 (0.91µM, 0.93µM) overexpression cell lines. With IC_{50} values of <1µM for HER2Δ16 cells, these two drugs are also more effective at inducing cell death compared to lapatinib with an IC_{50} >10µM. These results indicate the potential of either of these drugs to effectively inhibit HER2Δ16 action and thus combat the drug resistance seen in HER2Δ16 expressing tumors.

Task 5- Determine preclinical activity and specificity of novel HER2-targeting molecules: determine influence of targeting molecules on HER2 oncogenic signaling and cellular responses using multiple validated preclinical models of breast tumorigenesis and metastasis. (Months 12-24)
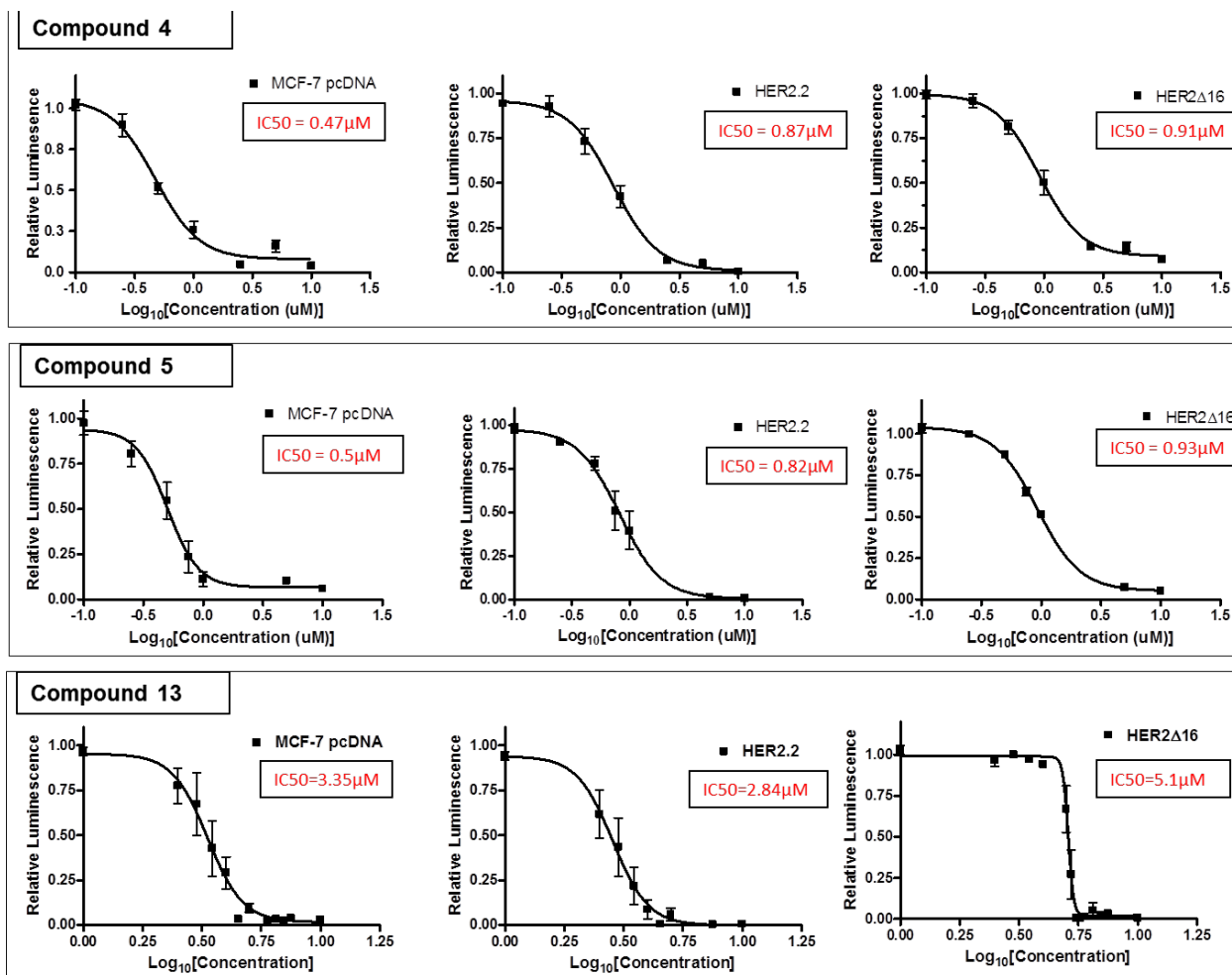
Figure 6: Inhibition of MCF-7/pcDNA, MCF-7/HER2 and MCF-7/HER2Δ16 cells by compounds **4, 5** and **13**.

Year 1: The compound NSC-322354 which showed the best inhibition activity against HER2Δ16 cell line was taken for an analysis of its cross kinase activity. The compound was sent to KinomeScan (www.kinomescan.com) for KINOMEscan's *in vitro* competition binding assay against a panel of 96 representative kinases. KINOMEscan™ is based on a competition binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active site-directed ligand. The assay is performed by combining three components: DNA-tagged kinase; immobilized ligand; and a test compound. The ability of the test compound to compete with the immobilized ligand is measured via quantitative PCR of the DNA tag (description of method taken from www.kinomescan.com). The compound was tested at a concentration of 10 mM. The results are given in Table 3. The S-score, selectivity score (the number of kinases that bind to the compound divided by the total number of distinct kinases tested), which is a quantitative measure of the compound selectivity, was 0.022. The compound showed good selectivity for two of the kinases, Casein Kinase 1 D and PIM kinases (more selective for PIM1 and PIM3 kinases). Both of these kinases are serine/threonine kinases. PIM1 is an oncogene. The PIM1 gene was initially identified as a proviral

Table 3. Matrix of Compound NSC322354 Screen

Kinase target	%Control @ 10μM		Kinase target	%Control @ 10μM
ABL1(E255K)-phosphorylated	96		KIT(V559D,T670I)	86
ABL1(T315I)-phosphorylated	95		LKB1	100
ABL1-phosphorylated	88		MAP3K4	100
ACVR1B	90		MAPKAPK2	93
ADCK3	100		MARK3	100
AKT1	100		MEK1	92
AKT2	100		MEK2	96
ALK	100		MET	100
AURKA	87		MKNK1	45
AURKB	72		MKNK2	98
AXL	100		MLK1	99
BMPR2	60		p38-alpha	100
BRAF	87		p38-beta	92
BRAF(V600E)	70		PAK1	93
BTK	100		PAK2	57
CDK11	86		PAK4	91
CDK2	92		PCTK1	100
CDK3	88		PDGFRA	100
CDK7	88		PDGFRB	100
CDK9	100		PDPK1	87
CHEK1	79		PIK3C2B	100
CSF1R	77		PIK3CA	99
CSNK1D	30		PIK3CG	68
CSNK1G2	100		PIM1	34
DCAMKL1	90		PIM2	67
DYRK1B	53		PIM3	36
EGFR	88		PKAC-alpha	84
EGFR(L858R)	78		PLK1	96
EPHA2	100		PLK3	54
ERBB2	93		PLK4	89
ERBB4	95		PRKCE	92
ERK1	100		RAF1	100
FAK	95		RET	100
FGFR2	100		RIOK2	86
FGFR3	93		ROCK2	77
FLT3	84		RSK2(Kin.Dom.1-N-terminal)	53
GSK3B	85		SNARK	90
IGF1R	100		SRC	91
IKK-alpha	87		SRPK3	93
IKK-beta	91		TGFBR1	100
INSR	97		TIE2	89
JAK2(JH1domain-catalytic)	94		TRKA	78
JAK3(JH1domain-catalytic)	61		TSSK1B	89
JNK1	96		TYK2(JH1domain-catalytic)	57
JNK2	84		ULK2	88
JNK3	76		VEGFR2	100
KIT	76		YANK3	89
KIT(D816V)	100		ZAP70	100

integration site in Moloney Murine leukemia virus-induced mouse T-cell lymphomas (Cuypers, H. T. *et. al.*, *Cell* **1984,** *37*, 141; Dhanasekaran, S. M. *et. al.*, *Nature* **2001,** *412*, 822). Pim kinases are implicated in the development of solid tumors. DNA microarray analyses showed the overexpression of PIM1 in human prostate cancer in relation to the grade of the prostate cancer. CK1d is a member of the ubiquitous casein kinase-1 family, and alterations in the expression and/or activity of CK1 have been observed in breast cancer (Giamas, G. *et. al.*, *Nucleic Acids Res.* **2009**, *37*, 3110). CK1d, has been identified as a novel kinase implicated in the modulation of physiological aspects of both ERa (estrogen receptor alpha) and AIB1 (amplified in breast cancer-1 protein). The compound in fact did not show good inhibition of HER2 (ERBB2) kinase. The *in vitro* high-throughput assay of the compounds is currently being performed for the proteins PIM1 kinase, and casein kinase 1 D. This will be followed by a dose response curve assay to determine the IC_{50} value for these kinases. These compounds will also be tested for inhibition of other breast cancer cell lines.

Year 2: A total of 40 compounds were subjected to high-throughput screening for inhibition of Pim1 kinase though an *in vitro* kinase assay. Out of these 9 compounds (Figure 7) showed notable inhibition potency ranging from 1.3 µM to 57.1 µM (Table 4). Four of these compounds were shown to inhibit the prostate cancer cell line DU-145 cells with inhibition potencies similar to that of emodin (Figure 8).

Figure 7: Structure of emodin and compounds **1** to **9** investigated for inhibition of Pim1 kinase
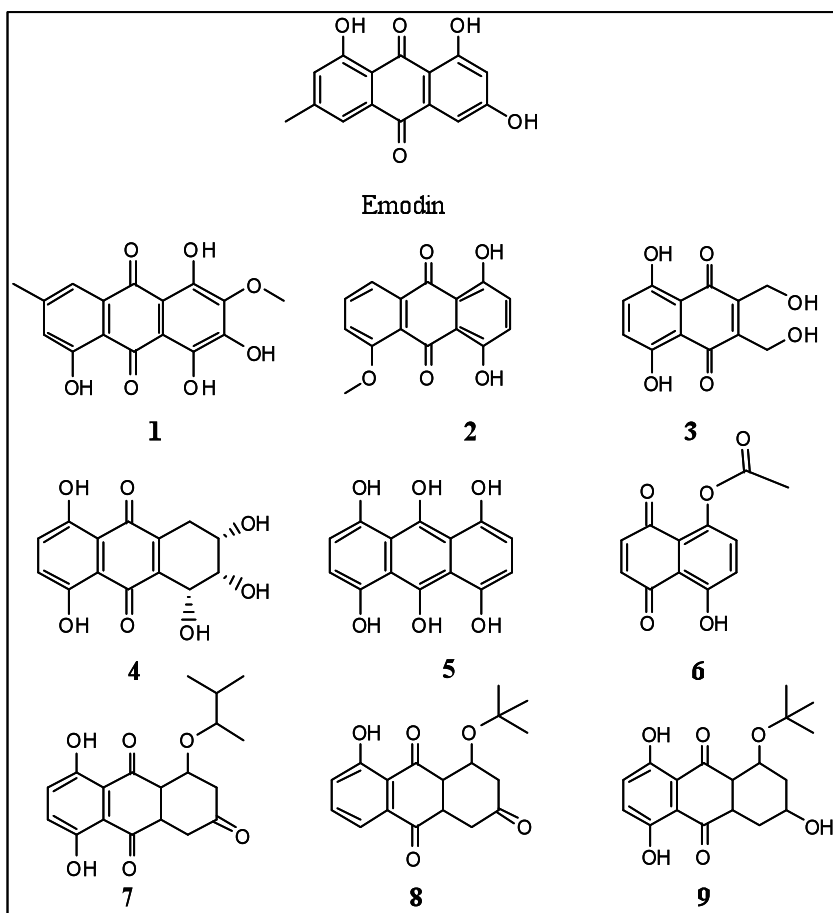
Compound	IC$_{50}$ (μM)
Emodin	2.5
1	21.4
2	11.6
3	7.4
4	19.2
5	57.1
6	1.3
7	3.6
8	23.8
9	3.5

Table 4: Inhibition of Pim1 kinase by emodin and compounds **1** to **9**.

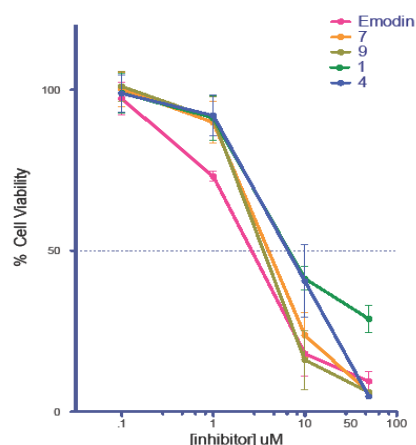**Figure 8:** Growth inhibition curve for DU-145 cells treated with compounds 1, 4, 7, 8 and Emodin.

5a - Perform genome wide shRNA library screen coupled with gene expression arrays of sensitive cells to identify Drug Targets, Drug Sensitizers, and Drug Resistance Pathways (Months 18-30)

Not initiated.

5b - Identify and validate drug combinations to improve efficacy and overcome resistance in preclinical models (Months 24-36)

Not initiated.

5c - Confirm efficacy of drug combinations in preclinical *in vivo* xenograft and transgenic mouse models of breast cancer (Months 30-48)

Not initiated.

Programmatic Activities

The breast cancer research group members have met multiple times to discuss and plan the various aspects of the project. A number of seminars/workshops were also held for the group:

1- On September 17, 2012, Dr. Matthew Burow presented a seminar/workshop to the group titled "Glyceollins – Development of Natural Product Based Anti-Estrogenic and Anti-Cancer Agents".

2- On September 24, 2012, Dr. Madhusoodanan Mottamal made a presentation to the group on the capabilities of the Xavier Molecular Structure and Modeling Core.
3- On October 1, 2012, Dr. Shafiq Khan from Clark Atlanta University made a presentation to the group titled "TGF-b Signaling During Different Stages of Prostate Cancer".
4- On November 12, 2012, Dr. Lucio Miele from the University of Mississippi Medical Center presented a seminar to the group tilted "Development of Notch Inhibitors in ER^+ Breast Cancer".
5- On March 4, 2013, Ms. Melody Baddoo from Tulane Cancer Center made a presentation to the group on the capabilities of the Tulane Cancer Center Crusaders Next Generation Sequence Analysis Core.
6- The group members participated and made presentations at the Louisiana Cancer Research Consortium Annual Retreat on March 8, 2013.
7- On April 26, 2013 the whole group (all faculty, staff, and students involved in the project) met for a pizza lunch at Tulane University Cell and Molecular Biology Department. This meeting was hosted by Dr. Frank Jones and his research group.

KEY RESEARCH ACCOMPLISHMENTS:

Foroozesh/Beckman/Burow Subproject

o Successfully developed a facile synthetic route to prepared 3-ketone-4,6-diene and 1-position-modified ceramide analogs, and obtained 16 novel ceramide analogs.
o Discovered a highly potent ceramide analog (406). The mechanism investigation showed that analog 406 leads to cell apoptosis through intrinsic apoptotic pathway and does not interrupt the function of GCS.
o Designed, synthesized, and determined a novel GCS inhibitor (503, a 1-position-modified ceramide analog), which is extremely useful for the development of highly potent GCS inhibitors.
o Successfully developed a facile synthetic route to prepare fluorescent building blocks for ceramide analogs. Eleven pyranoflavones and four 2-methylfuranoflavones were synthesized.
o Discovered the conformational isomerism of oxazolidine ceramide analogs. According to the evidences from NMR spectra, successfully constructed the molecular models of two conformational isomers.

Wiese/Burow Subproject

o Identified representative ER LBD structures to be used for virtual screening.
o Identified optimal ligand receptor (Docking) method for virtual screening.
o Obtained phytochemical and marketed drug databases for processing.
o Trained two pharmacy students, one in molecular modeling, and one in bioassays.

o Developed new, comprehensive method for virtual screening with ER LBDs.
o Trained one foreign exchange pharmacy student in bioassays.
o Obtained *in vitro* ER agonist and antagonist activity data for 29 naturally occurring stilbenes.

Sridhar/Jones/Stevens Subproject

o Identified three compounds that inhibit MCF7-pcDNA, MCF7-HER2Δ16 and MCF7-HER2 overexpressing breast cancer cell lines with sub-micromolar potency.
o Performed Western blots to detect total phosphorylated protein after treating each cell line with the three most potent drugs. IC_{50} values for the three compounds were measured for MCF7-pcDNA, MCF7-HER2Δ16 and MCF7-HER2 cell lines.
o Performed docking studies on the identified compounds revealing the two hydrogen bonds made by the quinones with the HER2 kinase. The first hydrogen bond made by all three compounds was to the hinge region residue Gln799. The second hydrogen bond was formed by the two compounds showing higher potency to one of the two residues- invariable Lys753 or Asp863.
o Discovered two new lead compounds for derivatization LM1 and LM2.
o Established synthetic protocols for the synthesis of different derivatives of LM1. The synthetic scheme for LM2 synthesis is presently being standardized.

Program Accomplishments

o Organized group meetings in order to introduce the students, staff, and faculty members working on the different subprojects to each other and the various projects.
o Organized training workshops/seminars.

REPORTABLE OUTCOMES:

o **Publications**

Foroozesh/Beckman/Burow Subproject

"3-Ketone-4,6-Diene Ceramide Analogs Exclusively Induce Apoptosis in Chemo-Resistant Cancer Cells", A. Ponnapakkam, J. Liu, K. Bhinge, B. Drew, T. Wang, J. Antoon, T. Nguyen, P. Dupart, Y. Wang, M. Zhao, Y.Y. Liu, M. Foroozesh, and B. Beckman, submitted to *the Journal of Medicinal Chemistry*, August 2013.

"A Review of Ceramide Analogs as Potential Anticancer Agents", J. Liu, B.S. Beckman, and M. Foroozesh, *Future Med Chem*, **5**, 1405-1421, 2013.

"Pyranoflavones: a group of small-molecule probes for exploring the active site cavities of cytochrome P450 enzymes 1A1, 1A2, and 1B1", J Liu, S. Taylor, P. Dupart, C. Arnold, J. Sridhar, Q. Jiang, Y. Wang, E. Skripnikova, M. Zhao, M. Foroozesh, *J Med Chem*, **56**, 4082-4092, 2013.

Wiese/Burow Subproject

None yet.

Sridhar/Jones/Stevens Subproject

"Identification of Quinones as HER2Δ16 and HER2 inhibitors for the Treatment of Trastuzumab Breast Cancer" Jayalakshmi Sridhar, Mary Sfondouris, Thuy-Linh Nguyen, Ian Townley, Cheryl Stevens, Frank Jones, to be submitted to the *Bioorganic Medicinal Chemistry Letters* in September 2013.

o **Presentations**

Foroozesh/Beckman/Burow Subproject

"The Design and Synthesis of Benzoate Esters as Potential Anti-proliferation Agents and Inhibitors of Cytochrome P450 Enzymes", C. Arnould, P. Dupart, J. Liu, and M. Foroozesh, the Annual LaSPACE Council Meeting, and the American Chemical Society Local Section Student Poster Presentation, New Orleans, LA, October 2012.

"Propargyl Flavones as Inhibitors of Human Cytochrome P450s 1A1, and 1A2", S. Taylor, J. Liu, P. Dupart, and M. Foroozesh, the American Chemical Society Local Section Student Poster Presentation, New Orleans, LA, October 2012.

"Quest for New Mechanism-Based Inhibitors of Cytochrome P450 Enzymes 1A1 and 1A2", J. Sridhar, J. Liu, M. Foroozesh, C.L. Stevens, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2013.

"Pyranoflavones and 5-Hydroxy-pyranoflavones as Small-molecule Probes into the Active Site Cavities of P450s 1A1 and 1A2", J. Liu, S. Taylor, P. Dupart, C. Arnold, and M. Foroozesh, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2013.

"Design, Synthesis and Evaluation of 3-Oxy-substituted Pyridine Terminal Alkynes", Q. Jiang, J. Sridhar, M. Minaruzzaman, J. Liu, and M. Foroozesh, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2013.

"Design, Synthesis, and Bioassays of Potential P450 Inhibitors", M. Foroozesh, Invited Oral Presentation at the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Quest for New Mechanism-Based Inhibitors of Cytochrome P450 Enzymes 1A1 and 1A2", J. Sridhar, J. Liu, M. Foroozesh, C.L. Stevens, the American Chemical Society

National Meeting, New Orleans, LA, April 2013.

"The Design and Synthesis of Benzoate Esters as Potential Anti-proliferation Agents and Inhibitors of Cytochrome P450 Enzymes", <u>C. Tutwiler</u>, <u>C. Arnold</u>, P. Dupart, J. Liu, and M. Foroozesh, the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Ethynyl Flavones as Inhibitors of Cytochrome P450 Enzymes", S. Taylor, J. Liu, P. Dupart, and M. Foroozesh, the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Flavone Derivatives as Small-molecule Probes of Cytochrome P450 Enzymes: Inhibitory Activity and Selectivity", J. Liu, S. Taylor, P. Dupart, <u>C. Arnold</u>, and M. Foroozesh, the American Association for Cancer Research Annual Meeting, Washington, D.C., April 2013.

Wiese/Burow Subproject

None yet.

Sridhar/Jones/Stevens Subproject

"Design and Development of Selective Pim1 Kinase Inhibitors" Jasmine Thompson, Ian Townley, Cheryl L. Stevens and Jayalakshmi Sridhar. ACS National Meeting, New Orleans, April 2013.

"Functionalization and modification of Shikonin compounds as HER2 inhibitors" Divya Jyothi Lella, Jayalakshmi Sridhar, Cheryl L. Stevens, Bangbo Yan. Western Kentucky University – Reach Week poster, April, 2013.

"Functionalization and modification of 2-hydroxymethyl-5, 8-dimethoxy-1, 4-naphthaquinone as HER2 inhibitors" Divya Jyothi Lella, Jayalakshmi Sridhar, Cheryl L. Stevens, Bangbo Yan. ACS National Meeting, Indianapolis, Sept 2013.

o **Employment or Research Opportunities**

Individuals trained in the first and second years of this DoD breast cancer project:

Jiawang Liu, Postdoctoral Fellow at Xavier University (Foroozesh Lab)

Jayalakshmi Sridhar, Postdoctoral Fellow at Xavier University (Stevens Lab, currently a new tenure-track faculty member at the Xavier University Department of Chemistry)

James Antoon, Medical Student at Tulane University (Beckman Lab, received his M.D. in May 2012 after receiving his Ph.D. in 2010 at Tulane University. He is currently doing his residency in pediatrics at the University of North Carolina.)

Barbara Drew, Medical Student at Tulane University (Beckman Lab, is currently in her residency in obstetrics and gynecology in Connecticut.)

Tony Wang, Medical Student at Tulane University (Beckman Lab, working on this DoD subproject)

Thong T. Nguyen, Undergraduate Student at Xavier University (Foroozesh Lab, graduated in May 2012 and is now pursuing a Ph.D. in Chemistry at Tulane University)

Adharsh P. Ponnapakkam, Undergraduate Student at Tulane University (Beckman Lab, graduated in May 2012 and is currently continuing his work on this DoD project as a Masters student at Tulane University)

Patrick Dupart, Technician at Xavier University (Foroozesh Lab, Xavier graduate, joined Virginia Commonwealth University as a graduate student in July of 2013)

Shannon Taylor, Technician at Xavier University (Foroozesh Lab, Xavier graduate)

Corey Arnold, Undergraduate Student at Xavier University (Foroozesh Lab)

Erika McClain, Undergraduate Students at Xavier University (Foroozesh Lab)

Brandon Dotson, Undergraduate Students at Xavier University (Foroozesh Lab)

Charne'sa Tutwiler, Undergraduate Students at Xavier University (Foroozesh Lab)

La'Nese Lovings, Technician at Xavier University (Foroozesh Lab, Xavier graduate)

Candace Hopgood, Technician at Xavier University (Wiese Lab, Xavier graduate, started Xavier Pharmacy School in August 2013)

Peng Ma, Technician at Xavier University (Wiese Lab)

Chioma Obih, Pharmacy Student at Xavier University (Wiese Lab, graduated in May 2013)

Gabriela Barbarini, Pharmacy Exchange Student at Xavier University (Wiese and Burow Labs)

Felicia Gibson, Pharmacy Student at Xavier University (Wiese Lab)

Elizabeth Martin, Graduate Student at Tulane University (Burow Lab)

Felicia Huynh, Graduate Student at Tulane University (Jones Lab)

Hope Burks, Graduate Student at Tulane University (Burow Lab)

Lucas Chan, Masters Student at Tulane University (Beckman Lab)

Lyndsay Rhodes, Postdoctoral Fellow at Tulane University (Burow Lab)

Melyssa Bratton, Instructor at Tulane University (Burow Lab, currently Research Associate at Xavier University Pharmacy)

Steven Elliott, Lab Supervisor at Tulane University (Burow Lab)

Van Hoang, Graduate Student at Tulane University (Burow Lab)

Han Wen, Graduate Student at Tulane University (Jones Lab)

Mary Sfondouris, Postdoctoral Fellow at Tulane University (Jones Lab)

Thuy-Linh Nguyen, Undergraduate Student at Xavier University (Sridhar lab)

Jasmine Thompson, Undergraduate Student at Xavier University (Sridhar lab)

Lella Divya Jyothi, Graduate Student at Western Kentucky University (Stevens lab)

Marleesa Bastian, Technician at Xavier University (Sridhar lab and is now pursuing graduation at Meharry Medical College school of Medicine, Tennessee)

CONCLUSION:

Foroozesh/Beckman/Burow Subproject

Our results have shown that extending the conjugated system in the backbone of ceramide analogs can lead to an increase in the anti-cancer activity. This observation is expected to assist us in designing more potent anti-cancer ceramide analogs.

We have also found that the modification of the 1-position of ceramides can lead to novel glucosylceramide synthase (GCS) inhibitors. This finding provides us with a new perspective for the design of effective GCS inhibitors.

In Year 2, we reported the methods for the syntheses of pyrano-/furano- flavones, and obtained 11 pyranoflavones and 4 corresponding furano derivatives, which are important building blocks of fluorescent ceramide analogs.

We have also discovered the conformational isomers of pro-apoptotic ceramide analogs, 401 and 402. This isomerism leads to the possibility that oxazolidine ceramide analogs could act on their molecular targets with two conformations. This finding

provides us with a new perspective for the investigation of ceramide-receptor interactions.

Wiese/Burow Subproject

In year one, we have developed the methods we will use to perform virtual screening of the phytochemical and marketed drug databases. This involved obtaining all crystal structures of the ERalpha ligand binding domain, sorting these structures by ligand type and structure characteristics, and then comparing and optimizing ligand receptor docking protocols. At the same time, we obtained the phytochemical and marketed drug databases, and started the process of filtering for compounds with potential to bind ER that will go into the virtual screening process. Two pharmacy students were trained and then involved in the molecular modeling as well as trained for the *in vitro* validation phase of the project.

In Year 2, we have developed a better way to sort and screen the two molecule databases based on the similarity of the database members to the bound ligands in all available ER LBD crystal structures. To carry out this method, training was obtained and a resorting of the databases is underway. Ligand-receptor docking methods have been evaluated and a standard protocol showing high performance in ER LBDs has been established. One pharmacy student and a new research assistant have been trained and are now generating bioassay data. A test set of 29 natural product stilbenes has been obtained and characterized for ER agonist and antagonist activity in a sensitive reporter gene system.

Sridhar/Jones/Stevens Subproject

Over the past two years we have been able to identify molecules that target two kinases, namely, PIM1 and CK1d, which play important roles in prostate cancer and breast cancer. Several compounds were found that inhibited MCF7 breast cancer cell line and HER2Δ16. Development of these lead compounds using molecular modeling and organic synthesis will give us potential drug candidates for breast cancer and prostate cancer. The dose response curve studies are ongoing for these two kinases. Based on the results further modification of the lead molecules will be attempted towards the goal of achieving better potency and selectivity for these two kinases. In the meantime, new database searches will be initiated based on the docking studies of known kinase inhibitors on HER2 to identify new core structures as lead molecules with the final goal of finding a new drug candidate for breast cancer.

Program

Other than the significant amount of scientific research performed and data collected during the first and second years of the project, it is important to note the valuable partnership developed between the two institutions involved. The productive collaboration formed between the Xavier University and Tulane Cancer Center

researchers participating in this program, once again proves the value and importance of inter-institutional research/training projects. The different training activities and the number of trainees involved in the various aspects of the subprojects also positively impact the future cancer research environment in the area. This breast cancer research project is still in its early stages and is expected to develop significantly over the next years.

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Wiese/Burow Subproject

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Sridhar/Jones/Stevens Subproject

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